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14. ABSTRACT
Recently, it has been recognized that a distinct mechanism of terminal proliferation arrest after chemotherapy involves the reactivation of senescence. However, whether this phenotype occurs in vivo is unclear, as is the biological impact of senescence induction. We have previously identified pathways and genes involved in human senescence that may serve as senescence markers, and have demonstrated that senescence occurs in prostate cancer cell lines after chemotherapy. In this proposal, we will: a) determine whether senescent tumor cells alter the proliferation and invasion of surrounding prostate cancer cells in vitro and in vivo, b) assess for and augment senescence in prostate cancer xenograft models and human tumors, and c) identify novel small molecules that induce senescence in prostate cancer cells. Both in vitro and in vivo approaches using human prostate cancer cells will be utilized to identify and determine the mechanisms underlying senescence. With this data, our understanding of cellular senescence will undergo a quantum leap and permit the translation of this entity both as a marker of response and for directing therapy.

15. SUBJECT TERMS
Androgen receptor, prostate cancer, DNA methylation

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Final Update and Report **12/14/2009**

Introduction:

Senescence is an irreversible process that limits the lifespan of normal cells. It is believed to represent a tumor-suppression mechanism that is lost during neoplastic transformation. The induction of accelerated senescence, like other damage responses such as apoptosis, is a programmed response to a carcinogenic or biological insult involving multiple molecular pathways. It has recently been appreciated that senescence may also be a cytostatic response *reactivated* in tumor cells in response to chemotherapeutic agents. A limiting factor in identifying and therapeutically exploiting this phenotype has been the lack of molecular markers. In the attached manuscript we present evidence for a panel of senescence-specific molecular markers upregulated in both replicative and induced senescence. We also demonstrate that induction of a senescent phenotype in prostate cancer lines using doxorubicin inhibits growth of untreated cancer cells. It is our **hypothesis** that the therapeutic activity induced by chemotherapeutic agents is due, in part, to a senescence-like program of terminal growth arrest. Furthermore, this phenotype inhibits the proliferation of surrounding cells and its presence may predict tumor response to therapy.

Body:

Task 1: **To determine whether senescent tumor cells alter the growth of surrounding prostate cancer cells *in vitro* and *in vivo***.

1. Co-culture and transwell experiments with ratios of senescent and proliferating cells; Generate senescent DU145 and LNCaP using DAC, doxorubicin and Docetaxel; proliferation and cell count; viablity (months 1-9)
2. Boyden chamber assays using ratios of senescent and proliferating DU145 and LNCaP cells (months 3-12)
3. *In vivo* studies using ratios of senescent and GFP-labeled non-senescent DU145 and LNCaP cells (10 animals per tx group; Total 50 for DU145 and 50 for LNCaP); GFP analysis cell count, BrdU proliferation, PI for viability, TUNEL/PARP for apoptosis. Statistical analyses (months 3-12)
4. If an effect on proliferation or invasion is seen then (months 6-24):
5. Repeat transwell and Boyden experiments with neutralizing antibodies to IGF receptors 1 and 2, (if stimulatory response) after western confirmation.
6. Repeat transwell and coculture experiments with neutralizing antibodies to IGFBP3 and 5(if inhibitory response)
7. Selective downregulation of putative effectors in senescent cells using siRNA

Completed.

This paper entitled "**Drug-Induced Senescence Bystander Proliferation In Prostate Cancer Cells *In Vitro* and *In Vivo*"** is attached (appendix 1) and has been published. The data and methods (Subtasks 1-6) are included within this manuscript. Additional data (unpublished) indicates that the inhibition of IGF2 prevents the proliferative bystander effect seen with the senescence phenotype (Figure 1). In conjunction with our previous data (Schwarze *et al* JBC, 2002) suggesting IGFBP3 is upregulated during senescence, this highlights the importance of this pathway in senescence induction.

Senescence is a distinct cellular response induced by DNA damaging agents and other sublethal stressors and may provide novel benefits in cancer therapy. However, in an aging model senescent fibroblasts were found to stimulate the proliferation of co-cultured cells. To address whether senescence induction in cancer cells using chemotherapy induces similar effects, we used GFP-labeled prostate cancer cell lines and monitored their proliferation in the presence of proliferating or doxorubicin-induced senescent cancer cells *in vitro* and *in vivo*. Here we show that the presence of senescent cancer cells increased the proliferation of co-cultured cells *in vitro* through paracrine signaling factors, but this proliferative effect was less than that seen with senescent fibroblasts. *In vivo*, senescent cancer cells failed to increase the establishment, growth or proliferation of LNCaP and DU145 xenografts in nude mice. Senescent cells persisted as long as 5 weeks in tumors. Our results demonstrate that while drug-induced senescent cancer cells stimulate the proliferation of bystander cells *in vitro*, this does not significantly alter the growth of tumors *in vivo*. Coupled with clinical observations, these data suggest that the proliferative effects of senescent cancer cells are negligible and support the further development of senescence induction as therapy. This paper has been supported in another recent publication by Di *et al.* (*Cancer Biol. Ther*, 2008).

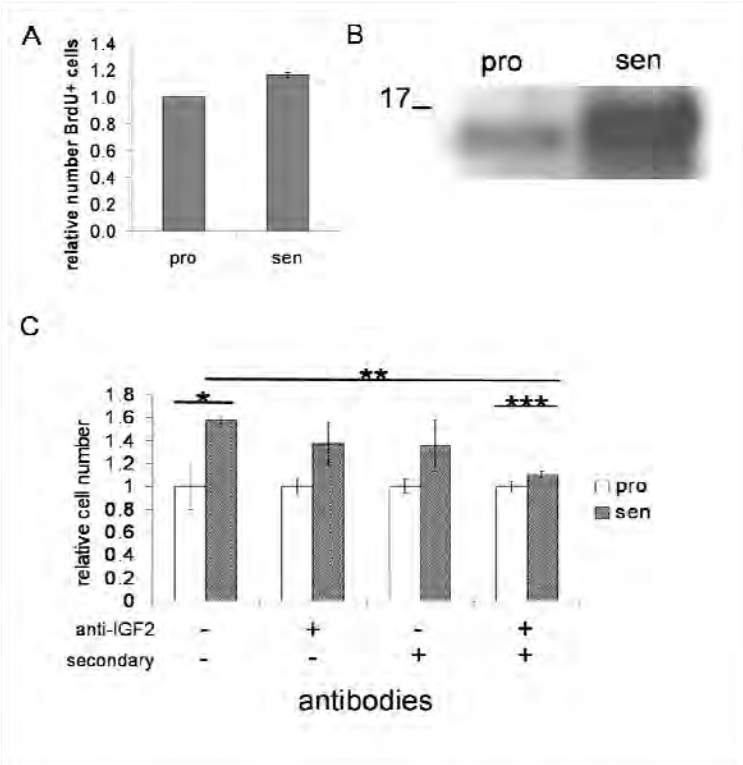

Figure 1: Involvement of secreted proteins in the senescent bystander effect in DU145 cells *in vitro*. (A) BrdU incorporation measured in proliferating DU145-GFP$^{(+)}$ cells co-cultured with proliferating (pro) or senescent (sen) cells in the lower transwell chambers. Three replicates for each experiment (3) were averaged and normalized to the data from cells co-cultured with proliferating cells ($p<0.0001$). (B) IGF2 protein expression in lysates of proliferating and senescent DU145 cells. 20 μg total cell protein in lysates was analyzed by western blotting with anti-IGF2. Specific bands between 10-30kDa (cleavage products) were seen to be increased in senescent cells. (C) Senescence induced proliferation is blocked by anti-IGF2 antibodies. Co-culture experiments were performed as in Figure 1, with anti-IGF2, anti-rabbit secondary or both in minimal media at total concentrations of 40 ng/ml each (1:5,000 dilutions). These results are representative of three independent experiments. Increasing anti-IGF2 or anti-rabbit antibody concentration 3 fold had no effect on proliferation (not shown). (*: $p=0.008$. **: $p<0.0001$. ***:

Task 2: **To assess for and augment senescence in prostate cancer xenografts and human tumor tissues.**

1. Generate Du145 and LNCaP xenografts in nude mice (months 6-24)
2. Treat with Docetaxel or doxorubicin and harvest at 3 intervals (3 intervals X 10treated/10control per xenograft line = total 60 for DU145 and 60 for LNCaP). GFP analysis cell count, BrdU proliferation, PI for viability, TUNEL/PARP for apoptosis (months 12-30)
3. QPCR and immunohistochemistry for senescence markers (months 12-36)
4. Analysis of human neoadjuvant tissues (10 treated/10 untreated per trial X 2). QPCR and immunohistochemistry for senescence markers (months 24-36)
5. Statistical analyses and correlation with proliferation

Completed:

Subtasks 1 and 2: We have set up these experiments and completed harvesting these mouse tumors. Nude mice (10 group) containing DU145 or LNCaP xenografts were treated with Docetaxol(10 mg/kg), Doxorubicin (5mg/kg), or vehicle on Days 0, 2, and 4 (3 experiments). BrdU pellets were implanted on the last treatment day. Animals were sacrificed 5 days after last dose. Tumors harvested for RNA, protein, sectioned for BrDU staining and SA B-gal. Treatment results are displayed in Table 1.

	DU145	**LNCaP**
Docetaxol	TV= -23% BrDU= -17%*	TV= -52% BrDU= -31%
Doxorubicin	TV= -33% BrDU= -35%	TV= -39% BrDU= -33% SA Bgal +

Table 1: Tumor volume (TV) and Proliferation (BrDU) in Prostate Cancer Xenografts Harvested after 4 days.

Subtask 3: Induction of SA-Bgal expression was only found with doxorubicin treatment. We additionally ran RNA for 9 senescence marker genes (Fu et al., Neoplasia, 2007) with the following significant induction results: Du145/Doxorubicin (1/9), LNCaP/Doxorubicin (8/9), Du145/Docetaxol (4/9), LNCaP/Docetaxol (1/9). We conclude that we are able to induce a growth inhibition using these drugs. Using LNCaP a senescence phenotype is found after treatment with doxorubicin. Docetaxol does not induce a robust senescent phenotype. No statistical correlation with proliferation was noted in the xenograft samples when proliferation was correlated with BrDU uptake. Further work with novel agents that induce senescence to a greater extent is detailed in Task 3 and more emphasis was placed on this approach since we concluded based on these studies that senescence induction utilizing these drugs was minimal.

Subtask 4: Given the lack of robust induction of senescent markers utilizing the majority of xenograft models and these drugs, we have had to modify our approach. We have been focusing on developing novel agents that induce a more robust senescence response (see below). Additional markers of senescence focusing on senescent-associated histone acetylation changes (HP1α, HP1γ, p27) and other published markers (IGFBP3, WNT2) are being utilized to determine if any are sensitive enough to reliably detect senescence in treated human tumors of epithelial origin.

Subtask 5: Completed.

Task 3: **To screen for small molecules capable of inducing senescence.**

1. Generate senescence reporter construct using CSPG2 and stably transfect prostate cancer cell lines DU145 and immortalized human prostate epithelial cell line HPV16E7. Select and test reporter. (months 1-6)
2. Optimization of detection conditions (months 6-12)
3. Screen 500 compounds with DU145 to gauge appropriate concentration
4. Screen full 16,000 compound library (months 12-18)
5. Secondary analyses of 25 most active compounds in other prostate cancer cells lines including QPCR for senescence markers, morphology, cell cycle arrest and SA B galactosidase staining. (months 18-30)

Completed. The paper entitled '**High-Throughput Screen to Identify Novel Senescence-Inducing Compounds'** has been published in JBS and is included in Appendix 1. The data and methods (Subtasks 1-5) are included within this manuscript. We had to undertake another approach to developing a screen for senescence-inducing compounds and based this on Hoechst fluorescence and SA-β-gal activity. Our initial approach (Subtask 1) included generating a reporter construct for *Cspg2* containing luciferase and transiently transfected it into the Du145 cell line. Unfortunately, when pooled transfectants were exposed to senescence-inducing doses of doxorubicin (25uM), we were unable to generate a reliable readout for senescence due to low expression levels. The failure of this approach lead to the idea that simply looking at cell number would allow an initial screen and this could be combined with SA-B-gal expression and morphology to screen for senescence induction.

Subtask 2-5: We developed a high-throughput, phenotypic screen to identify compounds in chemical libraries that induce the characteristics of cellular senescence in prostate cancer cells. DU145 was chosen as a model for advanced prostate cancer based on its androgen-independent growth, mutant p53 status, and ability to develop a strong senescent phenotype. The screen is based on the pairing of two compatible staining techniques; one that identifies growth inhibition, and the other SA-β-gal activity (Fig. 2A). The fluorescence of the DNA binding agent Hoechst 33342 was measured to determine cell number after compound exposure for 3 days. In validation studies, the average fluorescence of wells with proliferating cells versus cells induced to senescence with 25nM doxorubicin demonstrated an acceptable Z'-factor of 0.53. This screening-window coefficient indicates a high signal-to-noise and signal-to-background ratio. As this measurement does not differentiate between the induction of senescence or apoptosis, wells with low fluorescence were subsequently visually assessed for SA-*β*-gal staining and senescent morphology.

To identify senescence-inducing compounds, we screened a pilot library of 4160 known bioactive compounds and natural products (KBA) containing structurally diverse characterized compounds, drugs, pollutants and naturally occurring extracts. Using a dose of 10μM in a 96-well format, Hoechst 33342 staining resulted in 625 initial hits (Fig 2B). Compounds with fluorescence >1 standard deviation less than the average of "per plate" data were selected. Wells containing both SA-*β*-gal staining and a senescent morphology (51 compounds) were then assessed for their ability to induce a persistent growth arrest. In triplicate wells, cells were replated and exposed to each of the 51 compounds for 3 days, then allowed to recover following drug removal for an additional 3 days. Cells treated with 9 of the 51 compounds maintained their arrested growth state after removal of the drug (indicated by unchanged Hoechst 33342 intensity; data not shown).

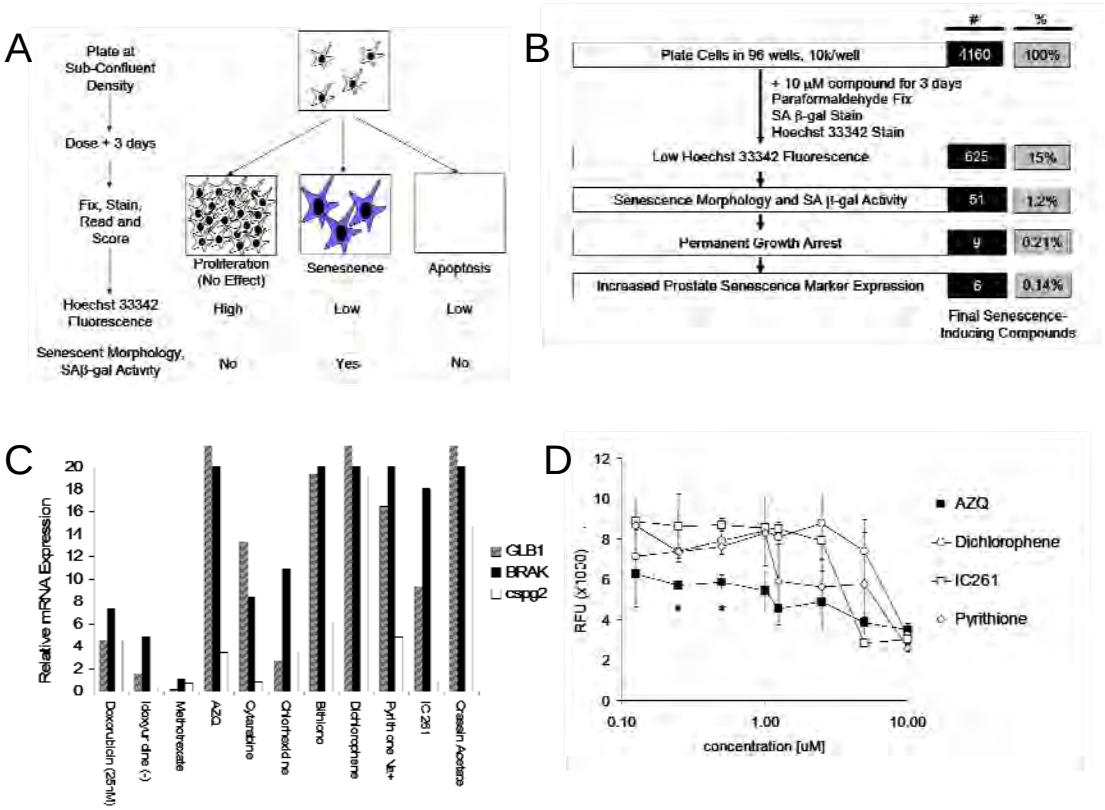

Fig. 2. Screen for senescence-inducing compounds. *A.* Du145 prostate cancer cells were plated on 96 well plates and utilizing robotic high-throughput screens, compounds from a library are plated. After 3 days, proliferation is determined by fluorescence after staining with Hoechst 33342. Low signal wells, indicating either senescence or apoptosis, were then visually examined for the presence of SA-β-gal staining and a senescent (enlarged, flattened) morphology. *B.* Results of the screen on a 4160 compound known bioactive compounds and natural products (KBA) library. Secondary tests included permanent growth arrest, and the induction of other senescent markers. *C.* Expression of senescence marker genes *GLB1, BRAK* and *cspg2* in DU145 cells treated with candidate or control compounds measured by qPCR and normalized to 18S expression. Doxorubicin (25nM) was utilized as a positive control [fu 2006], and one of several quiescence-inducing compounds (idoxyuridine shown) represents a negative control. Data is shown from one experiment performed in duplicate. *D.* AZQ inhibits Du145 cell growth at lower concentrations than other identified compounds. Hoechst 33342 fluorescence was measured after 3 days in wells after treatment with decreasing compound concentrations. Data showing chlorhexidine, bithionol, cytarabine and crassin acetate effectively inhibited proliferation only at doses higher than 1μM are are not shown for sake of clarity. These data represent the results of two independent experiments performed in triplicate. Error bars represent one standard deviation.

These 9 compounds were then tested to determine if they induce the expression of the previously identified senescence marker genes *Glb1*, *Brak* and *Cspg2*. After a 3 day compound exposure, qPCR was performed on RNA extracted from Du145 prostate cancer cells. Robust induction of all markers was demonstrated with 6 compounds (Fig. 2C) when compared to several quiescence-inducing controls (idoxyuridine shown). This experiment was reproduced using the hormone-dependent LNCaP prostate cancer cell line, confirming robust induction of all senescence marker genes with a final 4 compounds. In sum, this screen has identified compounds (Table 2), out of an original 4160, based on multiple previously established senescence criteria. These compounds are mechanistically diverse, and several had previously been identified as demonstrating growth inhibitory activity in cancer cells.

Table 1: Senescence-inducing agents identified by screening

Compound	PubChem ID	PubChem: Anti-Cancer Activity		Reported Mechanism of Action
		in vitro	*in vivo*	
AZQ	42616	+	-	DNA Alkylating
Bithionol	2406	+	-	N/A
Pyrithione	1570	+	N/A	Zn^{2+} Ionophore
Dichlorophene	3037	+	-	N/A

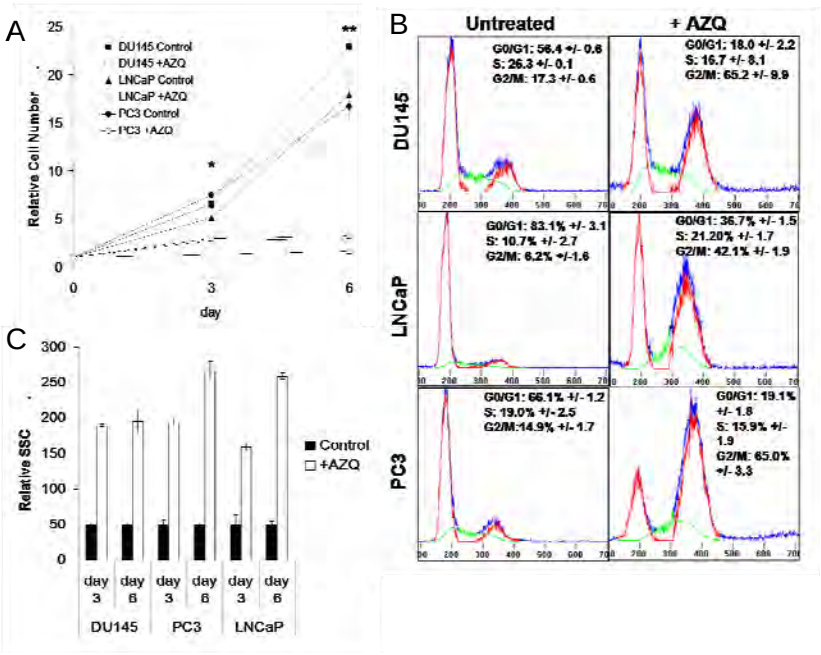

Figure 3: Exposure to senescence-inducing AZQ maintains viability. A. Viability of cells cultured -/+ AZQ, as measured by PI exclusion and cell size (forward scatter), normalized to data from untreated cell samples. (*: p< 0.03). Error bars represent standard error. B. Immunoblot analysis of full length PARP and a-tubulin expression in whole lysates of cells -/+ 250nM AZQ for 72 hr. These results are representative of three independent experiments. C. Detection of SAB-gal activity in whole DU145 cells cultured *in vitro* -/+ AZQ. Original magnification: 400x.

The Compound AZQ Induces A Potent Senescence Growth Arrest *In Vitro* and *In Vivo*. The relative potency of the identified compounds to inhibit cellular proliferation was tested. In 96-well plates, DU145 cells were treated with a range of compound concentrations (0.1-10uM) and the average well fluorescence measured after fixing the cells and staining them with Hoechst 33342 (Fig 2D). AZQ inhibited proliferation to a greater extent at sub-µM concentrations when compared to other identified compounds rendering it the most potent of these agents. Structurally, AZQ is a rationally-designed, lipophillic, DNA-alkylating quinone.

To demonstrate these results were not cell line specific, other prostate cancer cell lines were treated with AZQ and longer-term and complete growth inhibition was shown after drug removal (Figure 3A; p=0.01). Analysis of DNA content in cell lines at the 3 day timepoint shows that AZQ-treated cells accumulate in G2/M and are significantly different than untreated cells (p<0.0001; Fig 3B). The broad distribution of this peak suggests the possibility that this population may include cells arrested at late S phase checkpoints as well. A second characteristic of senescence, increased cellular complexity and size, was measured by flow cytometry using side-scatter(SSC)(10). SSC in viable AZQ-treated cells was increased in all cell lines at both 3 and 6 days (p<0.001; Fig 3C). Viability is another feature of

senescent cells. PI exclusion demonstrates all treated cell lines maintain an average of 71% +/- 4% viable cells after exposure to AZQ at both day 3 and day 6 timepoints when compared to untreated cells ($p < 0.03$; Fig 4A). Western analysis of protein lysates from AZQ treated cells were analyzed to evaluate apoptosis. Both proliferating and senescent cell lines maintain similar amounts of full length PARP without any detectable cleavage products that would be indicative of apoptosis(33) (Fig 4B). Given this and the cell cycle analysis data, the response of these cells to AZQ is largely non-cytotoxic.

Prostate cancer cell lines were then stained for SAB-gal activity, a marker of senescence \ and staining graded from 0 (no staining) to 3 (intense, complete staining). At 3 days after treatment, increased SAB-gal activity was demonstrated in treated cell lines (Fig. 4C).

Finally, we investigated whether AZQ induces a senescent phenotype *in vivo*. Previous studies had demonstrated in other tumor types an *in vivo* cystostatic response (25). As a model system, we generated DU145 xenografts roughly 1cm in size and treated them with a single intraperitoneal injection of 4 mg AZQ/kg body weight or vehicle (Fig 5). No toxicity was noted in the acute setting. Similar to *in vitro* results, increased SAβ-gal activity was observed in DU145 xenograft tumors of mice that were administered AZQ. By contrast, increased SAβ-gal activity was not observed in tumors from control mice injected with PBS vehicle (n=3). Apoptosis induction in these tumors, assessed using antibodies that specifically recognize cleaved PARP(33), showed minimal apoptosis in all tumors independent of AZQ treatment, suggesting that these molecular changes are not associated with apoptosis (data not shown). These

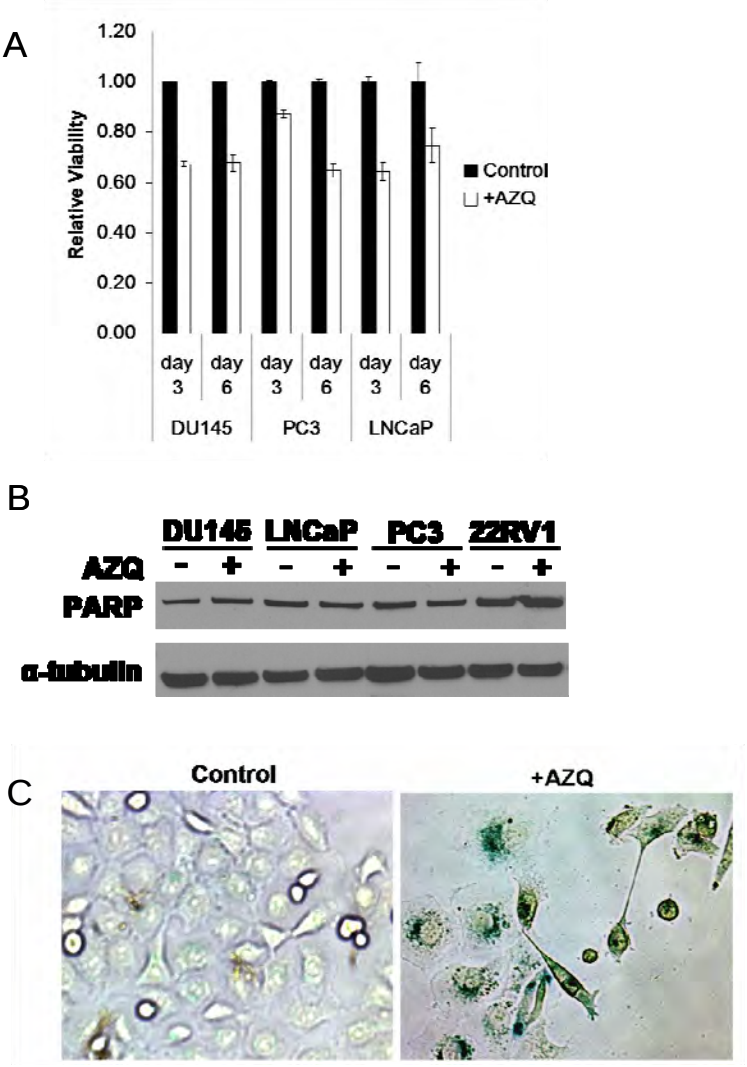

Figure 4. AZQ induces senescence without resulting in apoptosis. Prostate cancer cell lines were cultured in 250 nM AZQ or DMSO(control) for 3 days before drug removal. A. Viability of cells measured by PI exclusion and forward scatter using flow cytometry. Averaged data were normalized to untreated cell samples. A high proportion of cells remained viable in all samples. Error bars represent standard error. B. Immunoblot analysis of full length PARP demonstrates no induction of apoptosis in whole lysates of cells treated with 250nM AZQ for 72 hr. α-tubulin was utilized as a loading control. These results are representative of three independent experiments. C. Bright field microscopy demonstrates increased SA-β-gal activity in DU145 cells cultured with 250nM AZQ for 72 hr (original magnification 400x). SA-β-gal activity was similarly increased in PC3, LNCaP and 22RV1 cells cultured in 250nM AZQ (data not shown). These results are representative of four independent experiments.

results demonstrate the ability of AZQ to be effectively delivered *in vivo* and to induce SAβ-gal activity in DU145 prostate tumor xenografts.

In sum, AZQ to induce a phenotype consistent with senescence growth arrest. These data also validate the ability of our high-throughput screen to identify senescence-inducing compounds. Other compounds identified with the library have been published and are thus available in the public domain. AZQ is a putative compound being considered for a clinical phase I trial in a grant we are currently submitting.

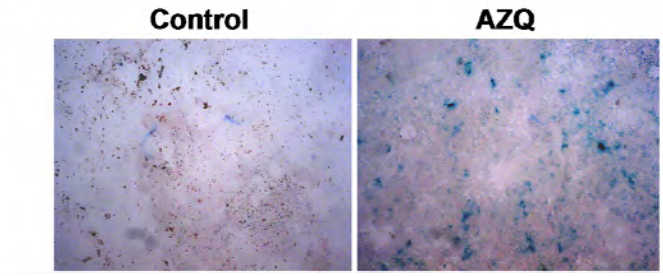

Figure 5: Bright field microscopy of SAB-gal activity in DU145 cells -/+ AZQ *in vitro*. These results are representative of 4 independent experiments.

The identification of this potent senescence inducing agent has permitted the investigation into mechanisms that are involved in senescence induction.

i) *Cyclin-dependent Kinase Inhibitor (CDKI) p27/kip1 is uniquely induced during cellular senescence in prostate cancer cells.* To examine genes involved in the cell cycle arrest seen at senescence, we evaluated a series of CDKI's for expression after treatment with AZQ. We demonstrate the induction of p27 is consistently induced in senescent cancer cells, as well as p21 in LNCaP and 22RVI (Figure 6). The expression of p27 was not induced at the RNA level suggesting a post-translational mechanism (data not shown). P27 has been demonstrated to induce senescence in cells when overexpressed(32). We had found previously that normal prostate cells undergoing replicative senescence induce p16 and p57(48), but these CDKIs are commonly inactivated in prostate cancer cells. P27 appears to represent an *alternate senescent block* that is activated in all cancer cells we have studied to date. Interestingly, p27 is commonly downregulated in prostate cancer, but is rarely deleted or mutated (49). *These data highlight that unique pathways induced during cellular senescence in cancer cells, and suggest an important role for p27 in the*

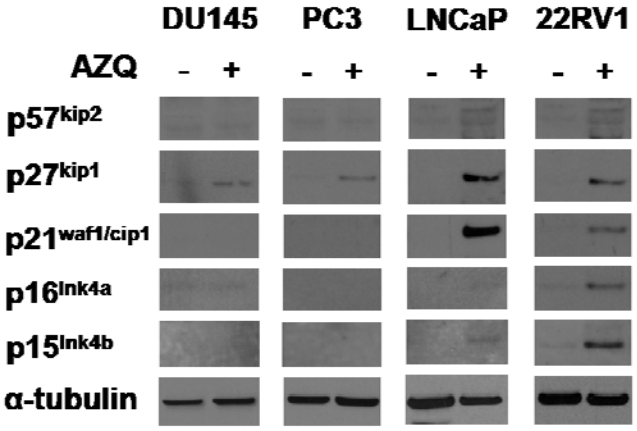

Figure 6. Evaluation of CDKI expression during cellular senescence. Prostate cancer cell lines were exposed to AZQ to induce senescence or control for 3 days. Western blot for CDKI's was performed. P27 was consistently induced in all cell lines. Notably, p27 RNA was not upregulated suggesting a post-translational mechanism for its regulation. P27 was also upregulated with low-dose Doxorubicin induced senescence (data not shown)

terminal arrest during induced in this phenotype. Given the decreased expression of p27 in human prostate cancers, and its marked induction in senescence, it provides a marker for following senescence *in vivo*.

vi) Ubiquitylation is selectively decreased during cellular senescence. To understand the increased expression of p27 (given a lack of mRNA induction) during cellular senescence in cancer cells, we evaluated ubiquitylation after AZQ and senescence-inducing (low) doses of doxorubicin. Global levels were initially evaluated by immunoprecipitation and western with two distinct anti-ubiquitin antibodies. Interestingly, no significant alterations were noted suggesting changes in ubiquitylation occur infrequently with senescence (data not shown). To investigate the increased p27 levels noted above with senescence, we performed immunoprecipitation utilizing p27 and assessed ubiquitin binding. In all cell lines, a significant decrease in ubiquitylated p27 was found during cellular senescence induced by AZQ (Figure 7B) and low-dose doxorubicin. P27 is a key substrate for an SCF (Skp1-Cullin1-F-box protein) ubiquitin ligase complex, which contains the F-box protein SKP2(S phase kinase-associated protein 2). SKP2 expression after AZQ treatment is consistently downregulated (Figure 7A). *This novel observation suggests that specific aspects of the ubiquitin pathway are altered during cellular senescence.* These may be exploited when considering therapeutic drug combinations for enhancing senescence. Further examination of this pathway will determine its importance in the induction of cellular senescence, and will also provide novel markers. *Notably, roughly 90% of prostate tumors overexpress SKP2(35) (and downregulate p27)(50;51) suggesting inducing senescence as therapy is particularly applicable to primary prostate cancers.*

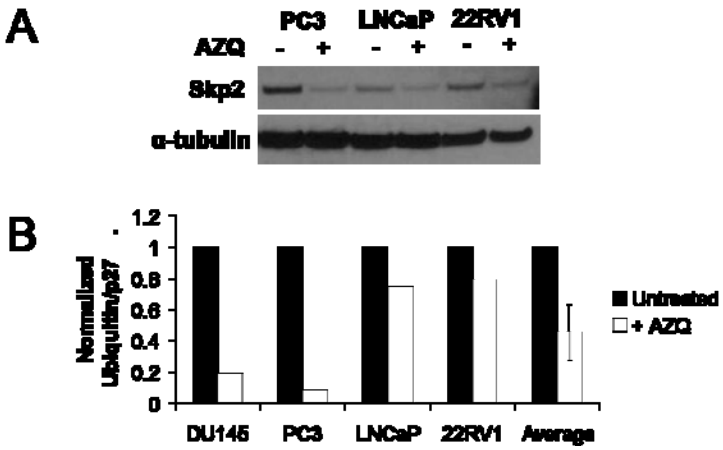

Figure 7. Ubiquination of p27 decreases during cellular senescence due to SKP2 downregulation. A) Skp2, a substrate for the SCF ubiquitin ligase complex is downregulated during cellular senescence in prostate cancer cell lines. B) Immunoprecipitation with p27 and western blotting with ubiquitin demonstrates that cultures treated with AZQ have less ubiquitylated p27. Similar results were seen with doxorubicin induced senescence.

Key Research Accomplishments:

- Senescence induces a bystander effect *in vitro,* but not *in vivo.*
- *In vitro* senescence is mediated, in part, by the IGF axis.
- A novel, whole-cell senescence screen has been developed that identifies novel agents that induce senescence robustly.
- Senescence is able to be induced in xenografts in vivo with specific agents.
- AZQ, a quinone, is identified as a novel senescence inducing agent.
- Docetaxol and doxorubicin induce senescence poorly in cancer xenografts
- Skp2-regulated p27 modulates senescence in cancer cells

Reportable outcomes:

Papers

1. Ewald JA, Desotelle JA, Almassi N and Jarrard DF. Drug-induced senescence bystander proliferating in prostate cancer cells in vitro and in vivo.. *Br J Cancer,* 2008.;98 1244-9.
2. Ewald JA, Desotelle JA, Laurila T, Almassi N and Jarrard DF. A Novel High-Throughput Screen To Identfy Senescence-Inducing Compounds. *J. BioMol. Screen.* 14(7): 853-858.
3. Ewald JA, Desotelle JA, Wilding G, and Jarrard DF. Cancer Cystostasis Using the Induction of Cellular Senescence (in review)
4. Ewald JA, Desotelle JA, and Jarrard DF. Drug-Induced Senescence in Prostate Cancer Cells Involves Skp2-Regulated $p27^{Kip1}$ Expression (in preparation)

Meetings and Abstracts

1. 'A Novel High Throughput Screen Identifies Potent Senescence-Inducing Activity of Diaziquone (AZQ) in Prostate Cancer Cells. Jonathan A. Ewald, Timo Laurila, Nima Almassi, Joshua A. Desotelle, and David F. Jarrard', American Association of Cancer Research Meeting, Washington DC, May 2008.
2. 'A Novel High Throughput Screen Identifies Potent Senescence-Inducing Activity of Diaziquone (AZQ) in Prostate Cancer Cells' Jonathan A. Ewald, Timo Laurila, Nima Almassi, Joshua A. Desotelle, and David F. Jarrard, American Urological Association meeting, Orlando FL, May 2008.
3. 'Androgen ablation generates phenotypic characteristics of senescence' Society for Basic Urologic Research meeting, Pheonix AZ, Nov 2008.

Final Conclusions:

While drug-induced senescent cells stimulate the proliferation of surrounding cancer cells *in vitro,* this does not significantly affect the longterm growth of bystander cells that might escape senescence induction. These data support further development of senescence-induction strategies for cancer treatment and is a key and novel finding funded by this DOD grant. Additionally, we have developed and validated a novel screen and find that senescence-inducing drugs are infrequently found in the libraries we have screened. These data do suggest that there are compounds that induce drugs specifically. This publication has generated a significant amount of interest from the scientific community. It provides a tool to develop novel senescence-inducing compounds for prostate cancer therapy, as well as providing further insight into mechanisms of senescence induction. Thus, this project has been successful in advancing the field of senescence and laying the further groundwork for its application in therapy.

A High-Throughput Method to Identify Novel Senescence-Inducing Compounds

JONATHAN A. EWALD,[1,2] NOEL PETERS,[2] JOSHUA A. DESOTELLE,[3] F. MICHAEL HOFFMANN,[2,4] and DAVID F. JARRARD[1-3]

Cellular senescence is a persistently growth-arrested phenotype in normal and transformed cells induced by noncytotoxic stress. Cytostasis as a method of cancer treatment has recently generated significant interest. Research into the induction of cellular senescence as cancer therapy has been hindered by a lack of compounds that efficiently induce this response. The authors describe a semiautomated high-throughput method to identify library compounds that induce senescence using prostate cancer cells cultured in 96-well plates. Primary hits are identified by low cell numbers after 3 days in culture, measured by Hoechst 33342 fluorescence. A secondary visual assessment of senescence-associated β-galactosidase staining and cellular morphology in the same wells distinguishes senescence from quiescence, apoptosis, and other false positives. This method was used to screen a 4160-compound library of known bioactive compounds and natural products at a 10-μM dose. Candidate compounds were further selected based on persistent growth arrest after drug removal and increased expression of previously described senescence marker genes. Four lead compounds not previously associated with senescence were identified for further investigation. This is the first successful assay to identify novel agents from compound libraries based on senescence induction in cancer cells. (*Journal of Biomolecular Screening* 2009:853-858)

Key words: cellular senescence, high-throughput screen, prostate, prostate cancer

INTRODUCTION

There is increasing interest in the development of oncostatic compounds that prevent the growth and progression of cancers without cytotoxicity. This strategy may increase patient survival while minimizing treatment side effects and chemoresistance in prostate and other cancers. The induction of cellular senescence is one mechanism by which this effect may be achieved.[1] Cellular senescence is a general program of persistent growth arrest in response to sublethal stresses in both normal nontransformed and immortalized transformed cells. Senescent cells cease dividing, become insensitive to mitogenic and certain apoptotic stimuli, and develop a phenotype similar to replicatively exhausted cells, exhibiting a characteristic enlarged and flattened morphology and increased senescence-associated β-galactosidase (SA-β-gal) staining activity (**Fig. 1A**).[1,2] Although ongoing studies seek to identify universal markers and regulators of senescence, SA-β-gal staining remains a standard and accepted marker used to identify senescent cells.

Agents that generate oxidative stress, DNA damage, and/or stress-related signaling induce cellular senescence. These include both endogenous processes, including telomere loss, accumulated oxidative damage, dysregulated oncogene activity, and exogenous factors such as chemicals, viral oncogenes, UV light, and ionic radiation. In aging organisms, cellular senescence represents an in vivo tumor suppressor mechanism that limits the proliferation of damaged cells.[1] This frequently involves the activity of tumor suppressors p53 and pRb and increased protein expression of cyclin-dependent kinase (CDK) inhibitors $p21^{waf1/cip1}$, $p16^{ink4a}$, and $p27^{kip1}$.[1] Cells exhibiting SA-β-gal staining and other senescence characteristics have been observed in benign lesions, including lung adenomas,[3] melanocytic naevi,[4] and prostatic intraepithelial neoplasia.[5] A similar senescent state can be chemically induced in prostate and other cancer cell lines in vitro, independent of p53, Rb, and other tumor suppressor pathways.[6,7] In humans, SA-β-gal staining has been observed in lung tumors[8] and breast tumors after treatment with genotoxic drugs.[9] Evidence in some studies suggests that the induction of senescence as a cancer treatment may benefit patients, including decreased incidence and severity of toxic side effects, stimulation of immune responses, and prolonged survival.[1,10,11] However, the investigation of drug-induced senescence in tumor models has been hampered

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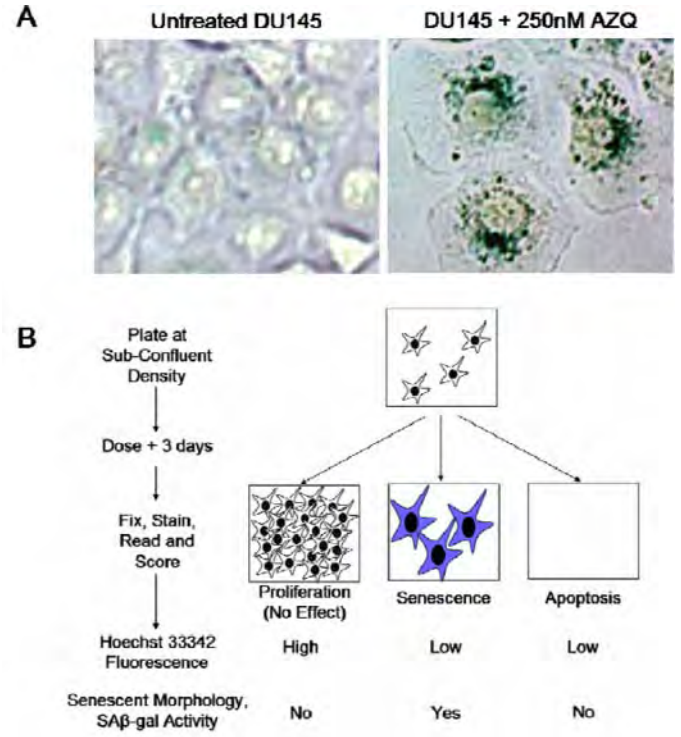

FIG. 1. Screen for the identification of senescence in cancer cells. (**A**) Senescent morphology and senescence-associated β-galactosidase (SA-β-gal) activity. Phase contrast microscopy of DU145 cells cultured with DMSO (control) or 250 nM diaziquone (AZQ), identified by this study, for 3 days, fixed and stained for SA-β-gal activity overnight, as previously described.[2] Original image magnification: 400×. (**B**) Multistep screening strategy. Prostate cancer cell lines were exposed to a library of compounds for 3 days and fixed and stained overnight for SA-β-gal activity, followed by staining with Hoechst 33342. Compounds of interest were initially identified by decreased Hoechst 33342 fluorescence indicative of low cell numbers. These wells were then visually assessed for the extent of SA-β-gal activity and senescent morphology.

by the lack of identified compounds that effectively induce this response.

Toward this end, we have developed a rapid semiautomated high-throughput method to screen libraries for novel compounds that induce senescence in prostate cancer cells. Cells are stained concurrently with DNA-binding Hoechst 33342 and for SA-β-gal activity, and compounds are selected on the basis of both growth inhibition associated with senescence and the phenotypic changes that result from its induction. Candidate compounds can then be further validated for induction of persistent growth arrest and expression of senescence marker genes. Using this assay, we screened a library of 4160 known bioactive compounds and natural products at a 10-μM dose, identifying 4 lead compounds not previously associated with senescence induction and demonstrating the utility of these methods.

MATERIALS AND METHODS

Compound library

Compounds used in this study were stored, maintained, and handled by the Keck-University of Wisconsin Carbone Comprehensive Cancer Center (Keck-UWCCC) Small Molecule Screening Facility (hts.wisc.edu/Index.htm). The compound library used for screening consists of 3 commercially available collections totaling 4160 compounds. This includes 2000 diverse Food and Drug Administration (FDA)–approved drugs and natural products (Microsource Discovery Systems, Gaylordsville, CT), the 1280-compound LOPAC^{1280} library of diverse characterized compounds (Sigma, St. Louis, MO), and 880 characterized compounds (Prestwick Chemicals, Illkirch, France). Compounds were dissolved in DMSO and stored in 384-well plates at –80 °C. Included on each 384-well plate are 64 DMSO negative controls. Further details can be obtained at http://hts.wisc.edu/Libraries.htm#kba. Compound structures were obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov).

Duplexed cell growth inhibition/SA-β-gal assay

Biomek FX robotic high-throughput fluid-handling instruments (Beckman-Coulter, Fullerton, CA) were operated by the Keck-UWCCC Small Molecule Screening Facility (hts.wisc.edu/Index.htm). DU145 cells were cultured as previously described,[7] suspended at a density of 1×10^4 cells/100 μL culture medium, and 100 μL/well added to 96-well plates (Corning #3906). Library compounds were administered to cells at a final concentration of 10 μM and incubated for 3 days. Cells were then washed in warm phosphate-buffered saline (PBS), fixed, and stained for SA-β-gal activity overnight, as previously described[2] using 100 μL/well. Cells were again washed in PBS and incubated at room temperature in PBS + 10 μg/mL Hoechst 33342 (Invitrogen, Carlsbad, CA) for 10 min. Hoechst 33342 fluorescence (ex/em: 355 nm/460 nm) was measured using a Victor V-3 high-throughput stacking plate reader (PerkinElmer, Waltham, MA). In control experiments, cells were cultured in medium containing 25 nM doxorubicin (Sigma) to induce senescence,[7] and Hoechst 33342 fluorescence was measured to calculate Z′ compared to proliferating cells, as previously described.[12]

In the pilot screen, wells in which fluorescence was more than 1 standard deviation (SD) below the average of 384 data points (1 drug plate) were then visually inspected by 3 independent observers to assess the intensity of SA-β-gal staining and the presence or absence of senescence morphology. Selected compounds were subsequently assessed for induction of persistent growth arrest by exposing cells to drug in 96-well plates for 3 days and then assessing growth by Hoechst fluorescence 3 days after drug removal. Doxorubicin (25 nM)-induced senescence arrest was used as a control and wells with Hoechst

fluorescence less than the average of the doxorubicin controls were visually inspected to confirm growth inhibition and development of a senescence-like morphology.

Prostate senescence marker gene expression

DU145 or LNCaP cells were cultured as previously described.[7] These cells were split to duplicate wells in 96-well plates at a density of 1×10^4 cells/100 μL culture medium and 100 μL/well and incubated overnight. Cells were then treated with 10 μM concentration of each selected compound and incubated for 3 days. RNA was isolated from cells and reverse transcribed, and *Glb1*, *BRAK*, *CSPG2*, and *18S* gene expression was measured by quantitative real-time PCR (qPCR) using an iCycler thermocyler and MyiQ software (BioRad, Hercules, CA) as previously described.[7] Expression of genes in each sample was standardized to 18S measurements, and relative expression of treated samples was normalized to that of untreated cells.

RESULTS AND DISCUSSION

Method development

Our larger interests lie in the characterization and development of compounds that induce senescence in prostate cancer models. However, this requires identification of compounds with this activity. To date, efforts to identify agents capable of inducing senescence have largely focused on testing individual compounds to determine the extent to which senescence is induced.[6] Here, we describe a high-throughput method to identify compounds in chemical libraries that induce characteristics of cellular senescence in prostate cancer cells.

Development of these methods presented numerous challenges. Although the importance of many molecular pathways regulating senescence induction has been described, these mechanisms may vary among cell types and are frequently inactivated in cancers.[1] Consequently, no standard universal markers of senescence beyond SA-β-gal staining/*Glb1* expression have been identified for use in cancer cells.[2,13] As androgen-independent DU145 cells develop phenotypic senescence and SA-β-gal staining in response to chemical treatment independent of p53 and Rb, these were used as a model of advanced prostate cancer.[7] However, although these cells were induced to senescence and increased SA-β-gal staining by exposure to 25 nM doxorubicin in control experiments, this did not significantly change the OD_{600} of whole or solubilized senescent cells when measured using a plate reader and thus by itself is not amenable to high-throughput screening (HTS; data not shown). Other attempts to identify senescence induction based on the expression of reporters regulated by the promoter of *CSPG2*, previously shown to be specifically upregulated in senescent prostate cancer cells,[7] were not significantly different in wells of proliferating or senescent cells.

Lacking a reliable individual marker of senescence, we ultimately adopted a multistep strategy based on identifying general phenotypic characteristics that define senescent cells—namely, the induction of persistent growth arrest, SA-β-gal staining, and morphological characteristics of senescent cells (**Fig. 1A**). Our method is based on the pairing of 2 compatible staining techniques that allow detection of growth inhibition and assessment of SA-β-gal activity in the same well. Cells are plated into 96-well plates, drugs are added, and after a 3-day incubation, they are fixed and stained for SA-β-gal overnight followed immediately by staining with the fluorescent DNA-binding Hoechst 33342 (**Fig. 1B**). Nuclear Hoechst 33342 staining can be measured in each well using a high-throughput plate reader to quickly identify wells with decreased fluorescence, indicative of low cell number. This dual staining did not interfere with either technique (data not shown). This relatively small set of wells could then be assessed visually to determine the extent of SA-β-gal staining, further selecting compounds for additional investigation.

Control experiments were performed to demonstrate the ability of Hoechst 33342 fluorescence to discriminate proliferating cells from senescent and apoptotic cells using increasing doses of doxorubicin. Exposure of DU145 cells to 25 nM doxorubicin had been previously shown to induce senescent cell morphology and SA-β-gal activity.[7] Our repeated experiments demonstrate that doses of doxorubicin 25 nM and higher reduce fluorescence significantly ($p < 0.003$) when compared to untreated or 5 nM doxorubicin after 3 days of exposure (**Fig. 2A**). We then compared the fluorescence of untreated proliferating cells to cells induced to senescence (**Fig. 2B**), and repeated experiments ($n = 4$) generated an average Z′ factor of 0.53, ranging from 0.5 to 0.6,[12] indicating a suitably high signal-to-noise ratio to identify growth inhibition using this technique. Increased concentrations of doxorubicin (100-250nM) induced apoptosis and cytotoxicity with low fluorescence similar to blank wells. Although the fluorescence of senescent cells (25 nM) is statistically different from higher cytotoxic doses ($p < 0.05$), the Z′ factor comparing these data was less than 0.5, indicating the need for additional analyses to distinguish senescence from cell death. Therefore, we used visual observation of SA-β-gal and cellular morphology to identify senescence in those specific wells that were growth inhibited.[2] Wells found to contain robust staining and senescent morphology (**Fig. 1A**) were selected for further assessment.

Pilot screen

We used this method to screen a library of 4160 known structurally diverse characterized bioactive compounds and natural products for senescence-inducing activity. After incubating cells with 10 μM of each compound or DMSO for 3 days, cells were fixed and stained and Hoechst fluorescence measured. Wells with a decrease in fluorescence greater than 1

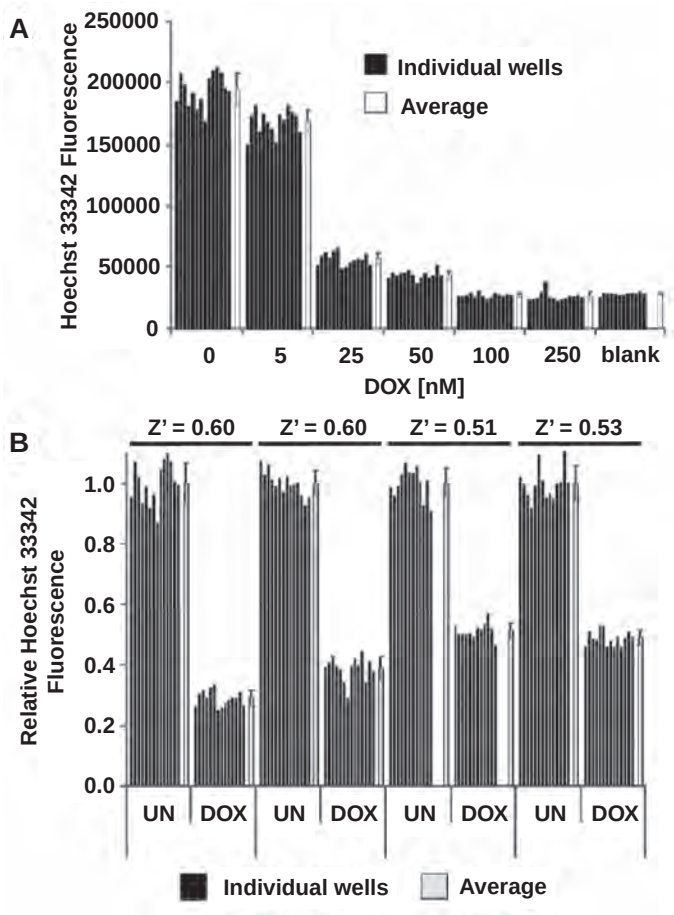

FIG. 2. Development of Hoechst 33342 fluorescence to identify senescence in treated cancer cells. (**A**) DU145 cells were cultured in a 96-well plate treated with increasing doses of doxorubicin (n = 14). Cells were cultured 3 days, fixed, and stained for senescence-associated β-galactosidase (SA-β-gal) activity and Hoechst 33342. Blank wells were included as negative controls. (**B**) Calculation of Z′ in senescent versus proliferating DU145 cells. Cells were cultured with 25 nM doxorubicin (DOX) or untreated (UN) for 3 days before being stained, and Hoechst 33342 fluorescence was measured by a plate reader. The average Z′ of all 4 experiments was 0.53 ($p < 0.003$). Error bars represent standard error.

SD of the average data resulted in 625 initial hits from the library (**Fig. 3A**). Subsequent visual scoring of these wells for robust SA-β-gal expression and senescence morphology identified 226 compounds as cytotoxic at 10 μM and 51 compounds as potentially inducing senescence (1.2% of the library).

Confirmatory assays

We tested whether these compounds induce a proliferation arrest in cells that persists after drug removal consistent with senescence. Cells were plated in duplicate wells, exposed to the 51 candidate compounds for 3 days, and then allowed to recover in drug-free media for an additional 3 days. After fixing and staining, we found that cells treated with 24 of the 51 compounds maintained decreased Hoechst 33342 fluorescence less than the average of cells cultured in 25 nM doxorubicin, used as a control for senescence induction (**Fig. 3B**). Visual assessment of these wells confirmed development of robust SA-β-gal staining and morphology induced by 9 compounds.

Glb1, *BRAK*, and *CSPG2* have been used as markers for induced cellular senescence in DU145 and other cancer cell lines.[7,13] We assessed expression of these genes in cells exposed to the 9 selected compounds. DU145 cells were treated for 3 days with 10 μM of each compound and gene expression analyzed by qPCR. Controls included untreated cells, and cells were exposed to idoxyuridine (a compound that induced a quiescent growth arrest). Cells induced to senescence with 25 nM doxorubicin were included as a positive control. Of the candidate compounds, methotrexate, cytarabine, chlorhexidine, and IC261 did not induce significant expression of all 3 markers (**Fig. 3C**). This experiment was reproduced using the androgen-dependent cell line LNCaP at the 10-μM screening dose (data not shown). These genes were similarly induced in this cell line by the remaining compounds except for crassin acetate. Finally, we confirmed that senescence induction by these remaining compounds does not induce apoptosis, based on the absence of both annexin V/propidium iodide staining and cleavage of poly(ADP)-ribosyl polymerase (data not shown).

Conclusions

In summary, we have developed these methods to screen compound libraries and identify those that induce senescence. Without ideal means to develop a completely automated screen, we developed a pragmatic semiautomated approach using a high-throughput first step followed by visual assessment of individual wells of interest, achieved by dual staining with Hoechst 33342 and for SA-β-gal activity. Consolidation of these 2 assays greatly reduces the time and resources required to make this initial identification, and although not completely automated, these methods are nonetheless rapid; a subsequent screen of 16,000 was completed within a month (data not shown). The diversity of compounds identified in the later stages of selection suggests the screen is not biased toward one particular pathway or mechanism. As understanding of induced cellular senescence in cancer cells continues to develop, reliable markers of senescence may be identified that both reliably identify senescence and are more amenable to HTS methods.

Our pilot screen of a known bioactive library at a single 10-μM dose identified 4 compounds not previously associated with senescence induction (**Table**). Several of these chemicals have demonstrated antiproliferative activity in cancer cell lines but have had limited in vivo testing. Mechanistically, diaziquone (AZQ) is a DNA alkylating compound with

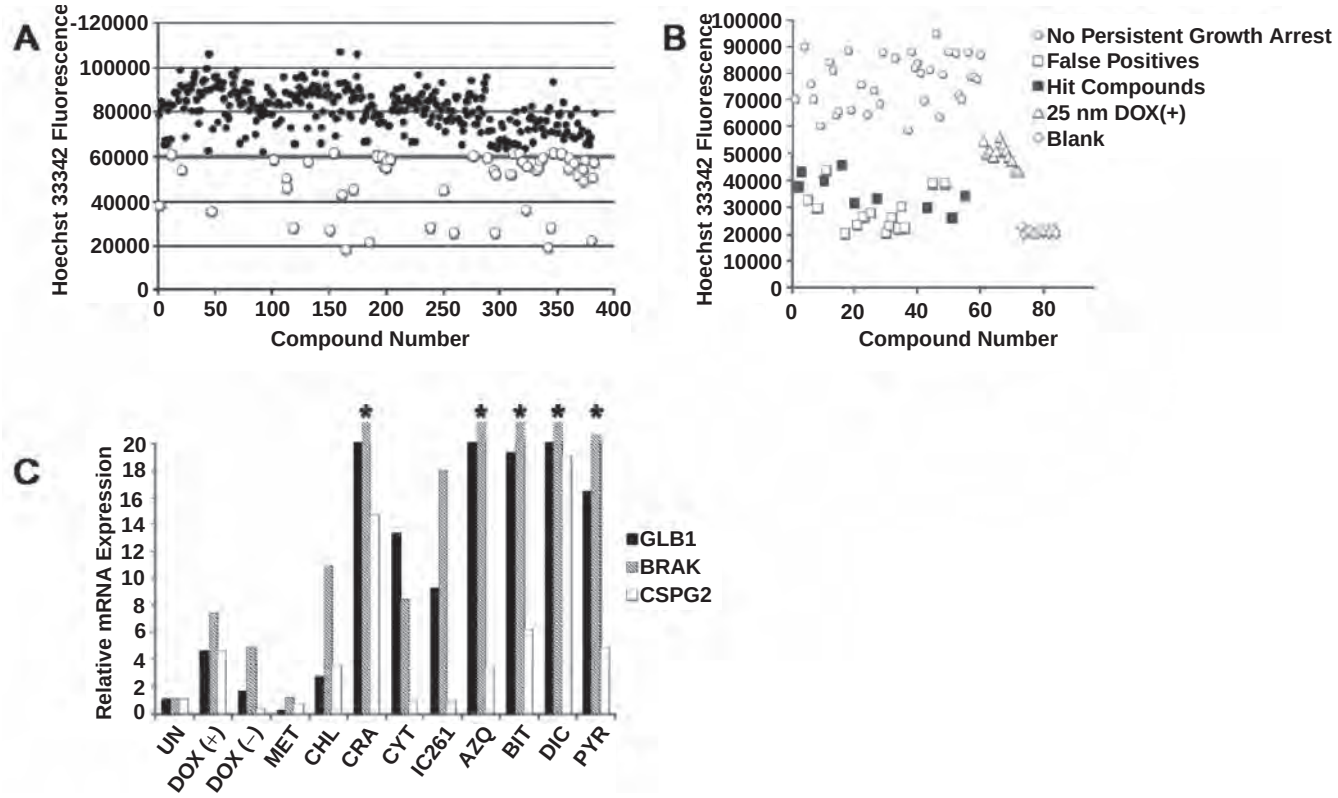

FIG. 3. Screen of known bioactive compound library and confirmatory assays for senescence induction. (**A**) Hoechst 33342 fluorescence measured in cells treated with library compounds or DMSO as a vehicle. Fluorescence data were analyzed in groups of 384 data points, and the average and standard deviation (SD) were calculated for each group. Wells with fluorescence decreased more than 1 SD below the average of all data (open circles) were selected to be visually assessed for senescence-associated β-galactosidase (SA-β-gal) activity and senescence morphology. (**B**) Persistence of proliferative arrest after drug removal. Wells with Hoechst 33342 fluorescence lower than the average of doxorubicin-treated cells were visually inspected, identifying 9 compounds that maintained senescence (closed squares). All data are the average of duplicates and include 12 untreated controls. (**C**) Expression of senescence marker genes *Glb1*, *BRAK*, and *CSPG2* in DU145 cells treated with 9 candidate compounds versus untreated. In triplicate wells, cells were left untreated (UN) or exposed to 10 μM of candidate compounds, 25 nM doxorubicin (DOX) as a positive control (+), and 10 μM idoxyuridine (IDO), which induces quiescence, as a negative control (–) and analyzed by quantitative real-time PCR (qPCR). Results were standardized to *18S* expression and then normalized to expression in untreated cells. Candidate compounds: methotrexate (MET), chlorhexadine (CHL), crassin acetate (CRA), cytarabine (CYT), IC261, diaziquone (AZQ), bithionol (BIT), dichlorophene (DIC), and pyrithione (PYR). These results are representative of 2 independent experiments. An asterisk (*) designates compounds inducing significant induction ($p < 0.05$) of all 3 markers.

limited cytotoxic activity in solid tumor models,[14] and the Zn^{2+} ionophore pyrithione induces oxidative stress.[15] Both of these cellular stresses are associated with senescence induction. The mechanisms by which bithionol and dichlorophene induce senescence remain unknown. From these, we have further characterized the senescence-inducing activity of AZQ in prostate cancer cell lines in vitro and prostate tumor xenograft models in vivo, the results of which are forthcoming (Ewald et al., in preparation). In addition, our screen identified more than 226 compounds in the library that are cytotoxic at 10-μM doses. As senescence induction has been described in response to low doses of cytotoxic compounds, these could be screened at lower doses to further identify senescence-inducing activity. In all, this development allows the advancement of investigations into the nature and regulation of induced cellular senescence in cancer cells and its utility as a means to treat and manage cancers.

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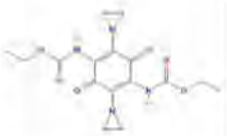
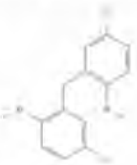
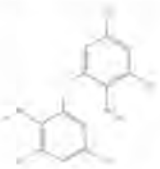
Table 1. Senescence-Inducing Compounds Identified by High-Throughput Screening

Compound Name		AZQ	Bithionol	Dichlorophene	Pyrithione
Structure					
PubChem ID		42616	2406	3037	1570
PubChem Anti-Cancer Activity	*In Vitro*	+	+	+	+
	In Vivo	-	-	-	Not Reported
Mechanism of Action		DNA Alkylation	Unknown	Unknown	Zn^{2+} Ionophore

(+) and (–) are indicative of a presence and lack of antiproliferative activity, respectively, as reported previously in PubChem and in the literature.

Due to constraints on the number of references in this manuscript, we apologize to authors of many other works that are pertinent to this investigation but could not be cited.

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Drug-induced senescence bystander proliferation in prostate cancer cells *in vitro* and *in vivo*

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Senescence is a distinct cellular response induced by DNA-damaging agents and other sublethal stressors and may provide novel benefits in cancer therapy. However, in an ageing model, senescent fibroblasts were found to stimulate the proliferation of cocultured cells. To address whether senescence induction in cancer cells using chemotherapy induces similar effects, we used GFP-labelled prostate cancer cell lines and monitored their proliferation in the presence of proliferating or doxorubicin-induced senescent cancer cells *in vitro* and *in vivo*. Here, we show that the presence of senescent cancer cells increased the proliferation of cocultured cells *in vitro* through paracrine signalling factors, but this proliferative effect was significantly less than that seen with senescent fibroblasts. *In vivo,* senescent cancer cells failed to increase the establishment, growth or proliferation of LNCaP and DU145 xenografts in nude mice. Senescent cells persisted as long as 5 weeks in tumours. Our results demonstrate that although drug-induced senescent cancer cells stimulate the proliferation of bystander cells *in vitro*, this does not significantly alter the growth of tumours *in vivo*. Coupled with clinical observations, these data suggest that the proliferative bystander effects of senescent cancer cells are negligible and support the further development of senescence induction as therapy.
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Keywords: senescence; bystander effect; prostate cancer; proliferation

Senescence is a physiological programme of terminal growth arrest occurring in both normal and immortalised cells in response to telomeric alterations, and also to sublethal stress and inappropriate oncogenic signalling. Senescent cells develop a characteristic phenotype, including an enlarged, flattened morphology, prominent nucleus, senescence-associated heterochromatin foci (SAHF), and senescence-associated β-galactosidase (SAβ-gal) activity (Narita *et al*, 2003; Campisi, 2005; Lee *et al*, 2006). Cancer treatments, including radiation and chemotherapy, induce senescent characteristics in cells. Doxorubicin and cisplatin are more efficient in generating senescence in cell culture than ionising radiation, etoposide or the microtubule-targeting drugs docetaxel and vincristine (Chang *et al*, 1999). Heterogeneous SAβ-gal staining has been observed in sections of frozen human breast tumours after treatment with cyclophosphamide, doxorubicin and 5-fluorouracil (te Poele *et al*, 2002), and in lung tumours, after carboplatin and taxol (Roberson *et al*, 2005). Senescence develops at lower drug concentrations than apoptosis, potentially limiting treatment-related side effects (Schwarze *et al*, 2005).

Senescence may provide a number of unique therapeutic benefits. When senescence is induced by expressing p53 in a murine liver cancer model, an upregulation of inflammatory cytokines triggers an innate immune response that targets the tumour cells (Xue *et al*, 2007). Other studies have suggested senescence may function as an alternative mechanism of tumour inhibition. In mice bearing Eμ-myc lymphomas, treated with cyclophosphamide, when apoptosis was blocked by Bcl-2 overexpression, senescence developed and these animals had improved survival over the apoptotic tumours (Schmitt *et al*, 2002). The recognition that a senescence programme may be reinduced in immortalised and tumorigenic cells by exposure to selected drugs presents a putative target for blocking cancer cell growth.

However, senescence induction may potentially promote tumour growth. Senescent cells express a variety of growth factors and secreted proteins that may stimulate as well as inhibit cell proliferation (Chang *et al*, 2002; Schwarze *et al*, 2002, 2005; Untergasser *et al*, 2002; Bavik *et al*, 2006). In contrast to apoptosis, a programme of cellular destruction, senescent cells persist and remain viable. SAβ-gal activity in cells has been putatively identified in ageing tissues, including skin and benign prostatic hyperplasia specimens (Dimri *et al*, 1995; Choi *et al*, 2000). Consistent with the hypothesis that ageing induces a procarcinogenic environment, fibroblasts passaged to replicative senescence induce the proliferation of local bystander cells both *in vitro* and in xenografts (Krtolica *et al*, 2001; Bavik *et al*, 2006). To determine whether senescent cancer cells generate a bystander effect or not, we chemically induced senescence in prostate cancer cells using doxorubicin and examined their effect on a bystander cancer cells *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Cell lines and cell culture

DU145 and LNCaP prostate cancer cell lines, and human primary fibroblasts, were cultured and senescence induced by treatment with 25 nM doxorubicin as described previously (Schwarze *et al*, 2005). Polyclonal green fluorescence protein (GFP)$^{(+)}$ cell lines were generated by infecting DU145 and LNCaP cells with pLS-GFP virus and repeated sorting of GFP$^{(+)}$ cells. Resulting cell lines stably express GFP in ~98 and ~80% of DU145- and LNCaP-derived cell lines, respectively. GFP$^{(+)}$ cells in both lines were approximately 100 × brighter than non-labelled cells, as measured by flow cytometry (data not shown).

Cell-counting experiments

For coculture experiments, 50 000 DU145 or 200 000 LNCaP GFP$^{(+)}$ tagged cells and equivalent proliferating or senescent untagged cells or 50 000 senescent primary prostate fibroblasts were plated together in triplicate in 35-mm wells containing growth medium. The following day, cells were washed twice in phosphate-buffered saline (PBS), given minimal medium (50% F12/50% DMEM + penicillin/streptomycin) and returned to 37°C, 5% CO_2. Cells were collected after 2 or 4 additional days in culture. Cell viability in counted samples was determined by annexinV binding (Invitrogen, Carlsbad, CA, USA) and by propidium iodide exclusion. Data were acquired from samples by flow cytometry and analysed using WinMDI v2.8 software (Joseph Trotter, Scripps Research Institute) to calculate the total number of viable GFP$^{(+)}$ cells in each sample.

Counting experiments were repeated using threefold the number of proliferating or senescent cells (from 50 000 to 150 000 cells), or a decreased fraction of senescent cocultured cells (75 and 25% senescent *vs* proliferating), incubated in minimal medium for 4 days and analysed as above.

BrdU incorporation

In cell-counting experiments (above), 20 mM BrdU was added to cell-culture medium, 30 min prior to trypsinisation, and GFP$^{(+)}$ cells were recovered by fluorescence-activated cell sorting. Isolated cells were fixed in 100% ethanol and stored at −20°C. Subsequently, cells were rehydrated and stained for BrdU as described previously (Krtolica *et al*, 2001; Schwarze *et al*, 2003). BrdU incorporation of cells cocultured in transwells did not require cell sorting.

Xenograft cocultures

All animal protocols and studies were conducted in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International, and approval was obtained from the University of Wisconsin Institutional Animal Care and Use Committee. Male athymic nude mice were obtained from Harlan (Madison, WI, USA). Xenograft tumours were established as described previously (Passaniti *et al*, 1992a, b). DU145-GFP$^{(+)}$ and unlabelled proliferating or senescent DU145 cells (0.5×10^6, each) were injected into the mouse subinguinal fat pad and allowed to develop into xenograft tumours over 5 weeks time. Tumour dimensions were measured at 3, 4 and 5 weeks after injection using a caliper. BrdU was injected into these mice interperitoneally at a concentration of 70 mg kg^{-1} body weight (Christov *et al*, 1993), harvested 2 h later and dissociated into a single-cell suspension from which GFP$^{(+)}$ cells were isolated by fluorescence-activated cell sorting. These were fixed in ice-cold ethanol and stored at −20°C. BrdU incorporation was measured in recovered cells, as mentioned above.

LNCaP xenografts were established by injecting 1×10^6 LNCaP cells alone, with 50% Matrigel (BD Biosciences, San Jose, CA, USA) or with an equal number of senescent LNCaP cells as described (Passaniti *et al*, 1992a, b), and cells were measured as mentioned above. Additionally, xenografts were established using 0.5×10^6 DU145 cells with or without addition of equal number of senescent GFP$^{(+)}$-DU145 cells. Tumours were measured as mentioned above, harvested at 3 and 5 weeks and samples were frozen in OCT for sectioning.

Immunofluorescence staining and microscopy

Ten micrometre sections of xenografts were fixed in PBS + 4% paraformaldehyde/0.2% Triton X-100/10 mM NaF/1 mM $Na3VO_4$ and washed in PBS + 0.2% Triton X-100/10 mM NaF/1 mM $Na3VO_4$ (wash buffer) before incubation in blocking buffer (wash buffer + 10% fetal bovine serum + 1% bovine serum albumin) for 1 h at room temperature. Sections were washed in blocking buffer and incubated with 1 μg ml^{-1} anti-IGF2 as a cellular counterstain (1 : 200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; no. sc-5622) overnight at 4°C. Sections were again washed, incubated for 1 h with 200 ng ml^{-1} (1 : 1000 dilution) anti-rabbit-Alexa 594 + 10 ng ml^{-1} Hoechst 33342 (Invitrogen), washed and mounted using ProLong Gold (Invitrogen). Images were captured using an Olympus microscope with mercury lamp, appropriate filters and spot digital camera and imaging software (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Images were merged and visualised using NIH ImageJ (http://rsb.info.nih.gov/ij/).

Statistical methods

Data were analysed, standard deviation and standard error were calculated, and Student's *t*-tests were performed using Microsoft Excel. Error bars in all figures represent one standard deviation in the data.

RESULTS

We generated stable GFP-expressing lines of the hormone-refractory DU145 (p53-inactive) and the androgen-dependent LNCaP (expressing functional p53) prostate cancer cell lines. To monitor the bystander effect of chemically induced senescent cancer cells, GFP$^{(+)}$ cells were cocultured with proliferating or senescent unlabelled cancer cells, collected and analysed by flow cytometry. Both DU145 and LNCaP cells treated with low-dose (25 nM) doxorubicin for 3 days develop a senescent phenotype, increased SAβ-gal staining (Figure 1A), and express previously described senescence marker genes (Schwarze *et al*, 2005).

Initially, DU145-GFP$^{(+)}$ or LNCaP-GFP$^{(+)}$ cells were plated with equal numbers of proliferating or doxorubicin-induced senescent untagged cells and cultured in a minimal serum-free medium for 2 and 4 days. GFP$^{(+)}$ cells cocultured with senescent cells were similar in number to those cocultured with proliferating cells at 2 days (Figure 1B). However, after 4 days, a significant increase in DU145 (1.46 fold; $P < 0.0001$) and LNCaP (1.51 fold; $P = 0.022$) cells was observed when cocultured with senescent cells. Apoptosis of GFP$^{(+)}$ cells, measured by annexin-V binding and propidium iodide exclusion at each time point, was not significantly affected by the presence of senescent cells (<1% in each sample), suggesting that these observed differences were not due to effects on cell survival. Proliferation, measured by BrdU incorporation, was also increased at day 4 (16–21%; $P = 0.003$) in GFP$^{(+)}$ DU145 cells, exposed to senescent cells (Figure 1C). When DU145 and LNCaP cells were cocultured in 0.4 m transwell inserts, preventing contact between the two populations but allowing exposure to common media, BrdU incorporation was similarly increased (20–24%; $P < 0.0001$ and $P < 0.05$, respectively). Given

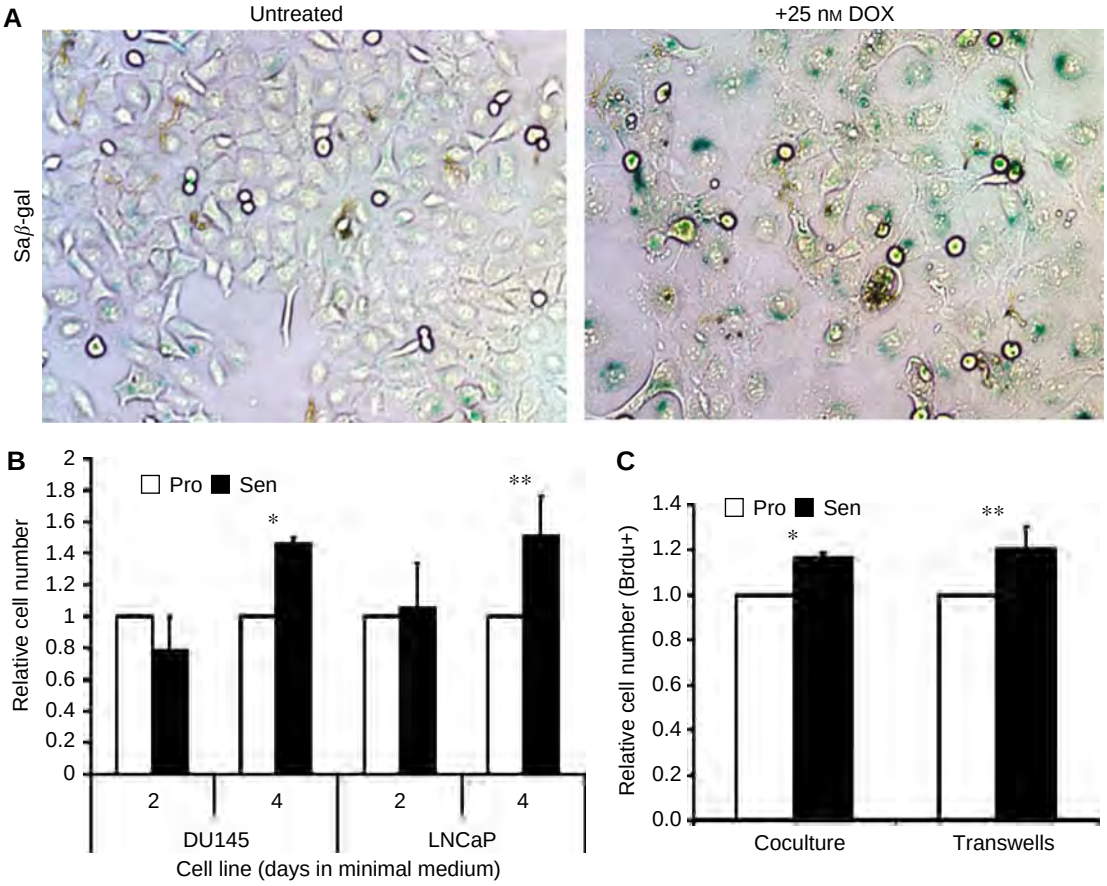


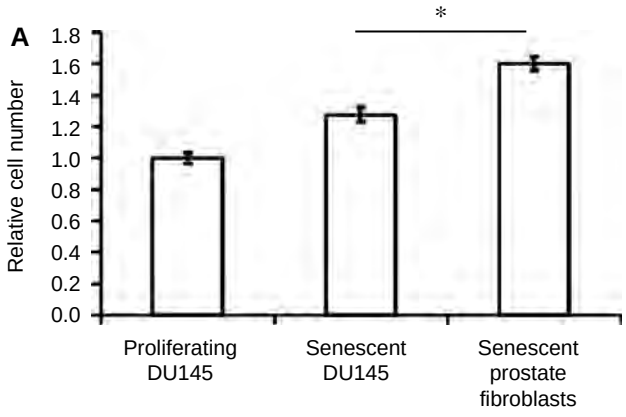
Figure 1 Proliferative bystander effect of drug-induced senescent prostate cancer cells *in vitro*. (**A**) Bright-field images of DU145 cells cultured on cover slips ± 25 nM doxorubicin (DOX) for 3 days, fixed and stained for SA β-gal activity (400 ×). (**B**) Number of proliferating DU145-GFP$^{(+)}$or LNCaP-GFP$^{(+)}$ cells after coculture with proliferating or senescent non-tagged cancer cells measured by flow cytometry. Replicate results were averaged from four independent experiments. These results represent the average fold increase of cell numbers in senescent cocultures relative to proliferative cell data. Error bars represent standard error (*$P<0.0001$; **$P=0.022$). (**C**) BrdU + incorporation in cells after direct coculture (left) and in transwells (right) after 30 min incubation with 20 uM BrdU. The results of three independent experiments were averaged and the numbers of cells from senescent cocultures were normalised to that of proliferating cocultures. Error bars represent standard error (*$P=0.003$, **$P<0.0001$).

the similar magnitude of this proliferative response to the mixing experiments performed on single plates suggested the majority of the growth stimulation observed was induced by secreted soluble factors.

Increasing the numbers of cocultured proliferating and senescent cells threefold in both DU145 and LNCaP cells (150 000 cells) sustained this proliferative response (1.4-fold; in both DU145 and LNCaP cells $P=0.03$ and $P=0.003$, respectively; data not shown) demonstrating that this effect was not an artifact of media depletion. Decreasing the fraction of cocultured senescent cells to 38 and 12% of the total cell population (decrease of 25 and 75% in the unlabelled senescent cells) did not induce proliferation (data not shown). These results demonstrate that a proliferative bystander effect can be stimulated *in vitro* by chemically induced senescent prostate cancer cells through paracrine signalling.

Previously published data have demonstrated a significant proliferative response of bystander cells to senescent fibroblast lines (Krtolica *et al*, 2001; Bavik *et al*, 2006). Therefore, we compared the proliferative bystander response of senescent DU145 cells to three replicatively senescent prostate fibroblast lines generated through prolonged passage in cell culture (Figure 2A). Senescent fibroblasts demonstrated SA-β gal staining and senescent morphology. After 4 days in coculture, the increase in the number of prostate cancer cells exposed to senescent fibroblasts was twice that seen with senescent cancer cells (60 *vs* 30%, respectively; $P<0.01$). We then confirmed the induction (>2 fold) of a number of growth-promoting paracrine factors in our chemically induced senescent DU145 and LNCaP cells (Figure 2B) using qPCR. No increase in expression of these genes (*IGF2, BRAK, FGF11* and *Wnt5a*) was seen in the senescent fibroblast lines. Comparing growth-promoting gene expression data from a number of studies involving fibroblasts, epithelial cells and cancer cells (Schwarze *et al*, 2005; Bavik *et al*, 2006) reveals little overlap when fibroblasts are compared to other cell lines (Figure 2B). In sum, our data show that senescent fibroblasts induce the proliferation of bystander cells *in vitro* significantly more than senescent prostate cancer cells.

Next, we investigated whether senescent cancer cells promote the growth of non-senescent cancer cells in nude mouse tumour xenograft models or not. LNCaP prostate cancer xenografts require additional growth factors, provided by Matrigel™, to establish viable tumours and proliferate (Passaniti *et al*, 1992a, b). To determine if senescence has a similarly permissive effect on xenograft tumour establishment, mice were injected with 1×10^6 LNCaP cells either alone, with 50% Matrigel or with 1×10^6 senescent LNCaP cells ($n=5$ in each group). Six weeks after injection, LNCaP cells coinjected with Matrigel developed into viable tumours in all five animals. In contrast, tumours did not develop under the other conditions (0/10 mice). This demonstrates that chemically induced senescent LNCaP cells do not promote tumour establishment and/or growth of this cell line.



B

Schwarze[1]	Bavik[2]
Senescent prostate cancer cells	Senescent prostate fibroblasts
*Igf2**	*Amphiregulin*
*Fgf11**	*CXCL1*
*Wnt5a**	*CXCL13*
*BRAK**	*CCL20*
MFAP2	*CCL2*
	Cytokine-like Protein C17
	FGF7
	HGF
	IL-8

Figure 2 Bystander proliferation induced to a greater extent by replicatively senescent prostate fibroblasts than senescent prostate cancer cells. (**A**) Number of proliferating DU145-GFP$^{(+)}$ cells cocultured with proliferating or senescent DU145 cells or three independent primary prostate fibroblast cell lines after passage to replicative senescence. Data from all three senescent fibroblast lines were averaged. Results are expressed relative to proliferating coculture data. Error bars represent standard error (*$P<0.01$). Results are representative of two experiments. (**B**) Expression of secreted growth factor genes reported in chemically induced senescent prostate cancer cells and senescent fibroblast ([1]Schwarze *et al*, 2005 *Neoplasia*; [2]Bavik *et al*, 2006. *Canc. Res.* *Increased gene expression confirmed in senescent cancer cells by quantitative RT-PCR in the present study).

Next, we examined the effect of senescent cells on tumour growth in DU145 xenografts using two different approaches. First, we coinjected 0.5×10^6 DU145-GFP$^{(+)}$ proliferating cells with an equal number of unlabelled proliferating or senescent DU145 cells (1×10^6 total) to model the effects of treatment-induced senescence in 50% of tumour cells. Tumours were palpable in both groups after 2 weeks and tumour dimensions were measured 3, 4 and 5 weeks after injection. The average volume of tumours established with or without senescent cells was calculated for each time point. Reflecting the greater number of proliferating cells initially injected, xenografts containing only proliferating cells grew significantly larger than those containing senescent cells after 5 weeks ($P<0.001$) (Figure 3A, left). However, the average exponential rate of tumour growth was not significantly affected by the presence of senescent cells, illustrated by calculating the natural log (ln) of the average tumour volume over time (Figure 3A, right). Control animals, in which only senescent cells were injected, did not develop palpable tumours through the course of these experiments. Mice were injected with 70 mg kg^{-1} body weight BrdU 2 h prior to tumour harvest to measure proliferation in sorted GFP$^{(+)}$ tumour cells (Christov *et al*, 1993). Cells from DU145 tumours established with or without senescent cells and collected after 5 weeks contain similar fractions of proliferating cells as measured by BrdU uptake and DNA profiling (data not shown). As a second approach, we repeated this experiment by beginning with equivalent numbers (0.5×10^6) of proliferating DU145 cells and determining the effect of adding additional (0.5×10^6) senescent cells. Again, the presence of senescent cells did not increase average tumour size or the rate of tumour growth (Figure 3B left, right).

Using senescent GFP$^{(+)}$-DU145 cells in this second approach allowed us to determine whether senescent cells persisted through the growth of these tumours or not. GFP$^{(+)}$ senescent cells were detected in xenograft tumours harvested 3 and 5 weeks after injection. However, at these time points, senescent cells were found infrequently (1–4 cells per section; mean 1 hpf; Figure 3C). SA β-gal analysis of tumour sections demonstrated infrequently stained cells, confirming these findings (data not shown). These results demonstrate that non-proliferating senescent cells become diluted during xenograft growth, yet persist even 5 weeks after injection. Therefore, the presence of chemically senescent cancer cells does not increase the rate of xenograft tumour establishment or growth *in vivo*.

DISCUSSION

Significant interest has been generated regarding the role of senescence as a tumour suppressor and the clinical ramifications of its reactivation in cancer (Schmitt *et al*, 2002; Petti *et al*, 2006; Xue *et al*, 2007). Potential exists for development of therapeutic compounds that specifically induce senescence in cancer cells (Roninson, 2003). However, concerns have been raised regarding the promoting effect of senescent cancer cells on the tumour microenvironment, similar to that seen with senescent fibroblasts (Kahlem *et al*, 2004). Our results demonstrate that a limited proliferative response occurs *in vitro* with chemically induced senescent cells when compared to senescent fibroblasts (Figure 2A). However, this bystander effect does not affect xenograft tumour establishment or the growth of non-senescent bystander tumour cells *in vivo* (Figure 3).

Using multiple cell types and combinations, senescent cells did not impact *in vivo* tumour growth or proliferation. When xenografts were established using proliferating cells with and without senescent cells, tumours were consistently smaller in the presence of senescent cells (Figure 3B). We acknowledge that a transient increase in proliferation may be induced prior to the development of a palpable tumour, but clearly, the long-term impact on tumour size was not significant. Technically similar mixing experiments in immune-deficient mice have been performed using senescent fibroblasts and a stimulatory effect was easily demonstrated using multiple immortalised and tumorigenic cell lines (Krtolica *et al*, 2001; Parrinello *et al*, 2005; Bavik *et al*, 2006). *In vivo,* these studies utilised equivalent numbers of proliferating and senescent cells similar to our methods. Our data clearly show the lack of a stimulatory response when senescent cancer cells are mixed with proliferating cancer cells in tumours. Furthermore, with current chemotherapy regimens, senescent cells appear at a much lower frequency (<20%) than those tested in our experiments (te Poele *et al*, 2002; Roberson *et al*, 2005).

As part of our study, we contrasted, *in vitro*, the bystander effect of senescent fibroblasts to that seen with chemically induced senescent cancer cells. Using our quantitative and reproducible model, the *in vitro* proliferative effect of senescent cancer cells was noted to be 40–50% of that seen with senescent fibroblasts. Our

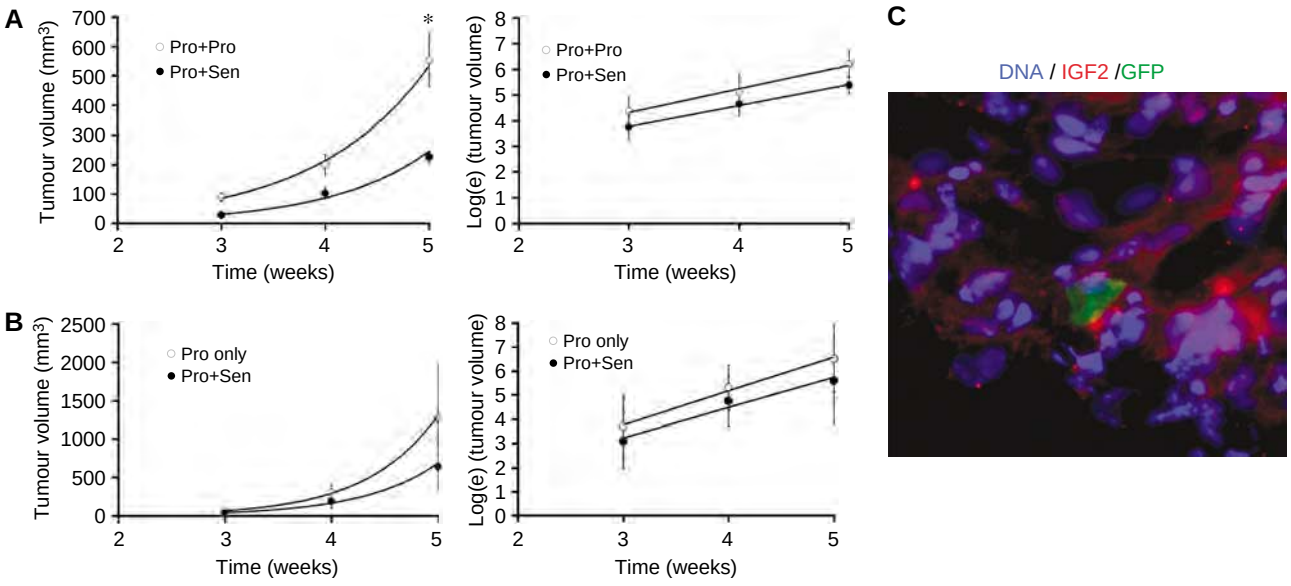


Figure 3 Xenograft tumour growth is not promoted by senescent DU145 cells. (**A**) Average size (left) and natural log of tumour size (right) of prostate xenograft tumours established using DU145-GFP$^{(+)}$ cells (0.5×10^6) mixed with an equal number proliferating (Pro + Pro) or senescent (Pro + Sen) cells and measured for 5 weeks. Error bars represent standard error (*$P<0.001$). Fit equations: (left) (Pro + Pro): $y = 5.491e^{0.913x}$ ($R^2 = 0.996$); (Pro + Sen): $y = 1.362e^{1.039x}$ ($R^2 = 0.981$); (right) (Pro + Pro): $y = 0.917x + 1.565$ ($R^2 = 0.989$); (Pro + Sen): $y = 0.813x + 1.340$ ($R^2 = 0.997$). (**B**) Average size (left) and natural log of tumour size (right) of prostate xenograft tumours established using DU145 (0.5×10^6) cells alone (Pro only) or with an equal number of additional senescent GFP$^{(+)}$-DU145 cells (Pro + Sen). Error bars represent standard error. Fit equations: (left)(Pro only): $y = 0.739e^{1.491x}$ ($R^2 = 0.999$); (Pro + Sen): $y = 0.576e^{1.417x}$ ($R^2 = 0.994$); (right)(Pro only): $y = 1.41x - 0.458$ ($R^2 = 0.994$); (Pro + Sen): $y = 1.254x - 0.527$ ($R^2 = 0.966$). (**C**) Xenograft tumour section containing a senescent GFP$^{(+)}$-DU145 cell. Hoechst 33342 (blue) was used to stain nuclei and anti-IGF2 (red) was used to stain cytoplasm (400 ×). This image is representative of sections from 10 xenograft tumours containing 1–4 GFP$^{(+)}$ cells per section (mean: 1 hpf).

quantitative PCR analysis confirmed the results of studies that show significant variation between growth-promoting genes expressed by senescent epithelial cells, fibroblasts and cancer cells (Chang *et al*, 2002; Schwarze *et al*, 2002, 2005; Untergasser *et al*, 2002; Zhang *et al*, 2003; Bavik *et al*, 2006). The finding that gene expression perturbations during senescence differ greatly between fibroblasts and epithelial cells, but show physical clustering on DNA, has been thought to reflect the altered chromatin structure seen during senescence (Zhang *et al*, 2003). These changes are likely to be even more marked in cancer cells containing deletions, duplications and distorted nuclear structure.

In vivo, the expression of secreted extracellular matrix, growth factors and surface receptor proteins differs markedly from cells cultured *in vitro* (Gieseg *et al*, 2004). This disparity in the tumour microenvironment may contribute to the lack of induction of proliferation in response to senescent cells *in vivo*. As an example, IGF2 protein expression is clearly elevated in senescent cancer cells *in vitro,* but the expression of IGF2 protein does not quantitatively differ *in vivo*, when senescent and proliferating cells are compared (data not shown). A unique aspect of our study is the demonstration of a persistence of senescent cells in tumours as long as 5 weeks after injection. They represent a small population at this time point, less than 1%, due to expansion of the proliferating population, which doubles in roughly 48 h (Passaniti *et al*, 1992a, b). Senescent cells have been noted in the skin of elderly individuals (Dimri *et al*, 1995) and in melanocytic naevi (Michaloglou *et al*, 2005). Our data in a xenograft model would support the persistence of these cells in various organs.

Placing senescence induction in the context of cancer treatment, our results suggest that the specific induction of senescence in prostate tumour cells would not promote tumour growth. Accumulating data suggest that senescent cells may occur *in vivo* after the treatment of tumours with chemotherapy, in approximately 40% of breast tumours after treatment using a CAF regimen (te Poele *et al*, 2002). Other observations support that senescence *in vivo* is a beneficial phenotype by inducing a cellular immune response (Petti *et al*, 2006; Xue *et al*, 2007) and demonstrating a survival advantage when compared to solely apoptotic responses (Schmitt *et al*, 2002). Recently, senescent cells were identified in human melanocytic nevi, a benign, stable skin lesion, supporting its function as a long-term tumour-suppressive mechanism (Michaloglou *et al*, 2005). In this case, there are no apparent signs of enhanced bystander proliferation or increased local carcinogenesis. Staining for senescent cells has also been identified in benign prostatic hyperplasia tissues, a common benign entity not associated with cancer (Choi *et al*, 2000). In conclusion, our data demonstrate that the presence of chemically senescent prostate cancer cells does not significantly enhance the growth of tumour xenografts, providing further rationale for the development of anticancer strategies that efficiently induce senescence in advanced cancers.

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Cancer Cytostasis Using the Induction of Cellular Senescence

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Abstract

There is an emerging interest in cytostasis, or the arrest of tumor growth, as an alternative to cytotoxic strategies for cancer treatment. Cellular senescence is a distinct response to non-lethal stress that results in a persistent cytostatic phenotype. Senescence can be induced in immortal and transformed cancer cells by selected anti-cancer compounds or radiation. This phenotype can be generated in cancer cells lacking functional p53, Rb and disrupted apoptotic pathways. Accumulating data indicates therapeutic senescence has reduced toxicity-related side-effects, increased tumor-specific immune activity, and prolongs survival when compared to classical chemotherapeutic approaches. Senescence can be detected in tumors through the expression of specific mRNA and protein markers. These may be valuable in predicting outcomes in both untreated and post-treatment patient tumors. These findings suggest cytostasis induced by senescence represents a novel functional target that may lead to improved cancer therapy. The identification of additional compounds and other means to induce senescence in tumor tissues will further allow development of therapeutic senescence in the clinical treatment of cancer.

1. Introduction

Cancer therapy has traditionally relied on cytotoxic treatment strategies based on the assumption that complete cellular destruction of tumors optimizes the potential for patient survival. This view has limited the treatment options that oncologists have at their disposal to toxic compounds and high dose radiation that infrequently produces complete cell death within a solid tumor. Often such cancers develop resistance to treatment, recur and progress to advanced primary and metastatic tumors. At the same time, this approach can cause severe side effects in patients. An alternative strategy involves permanently disabling the proliferative capacity of cells without necessarily inducing cancer cell death, inducing a state of "cytostasis". In theory, this approach to treatment could provide equivalent or prolonged survival with fewer and less severe side-effects related to cytotoxicity. Simply, it may be as effective to prevent continued tumor growth as it is to attempt to absolutely kill tumors. This may provide a more realistic goal for the chronic management of some cancers. Initial clinical studies utilizing cytostatic treatments have yielded promising preliminary results (1-3).

One potential means to induce cytostasis in tumor cells is by therapeutically stimulating cancer cells to become senescent (4). *Replicative senescence* was first described as a phenotype in primary cells after extensive culture and replicative exhaustion *in vitro* that is linked to telomere shortening(5, 6). More recently, DNA damage and other genomic stress, increased oncogenic signaling, and oxidative stress were found to result in *induced* or *accelerated senescence.* Senescent cells remain viable and metabolically active, but are persistently growth-arrested and insensitive to mitogenic factors and selected apoptotic stimuli (7). Growth arrest is achieved and maintained, in part, by the increased expression of specific cyclin-dependent kinase inhibitors (CDKIs) including $p16^{Ink4a}$, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (8). It is also recognized that transformed, neoplastic cells, including those lacking p53 and other tumor suppressor genes, retain this capacity to become senescent when exposed to certain stresses, including those generated by selected anti-cancer agents and ionizing radiation (9-11).

Cultured *in vitro*, senescent cells develop a distinct and easily recognizable flattened and enlarged morphology with a prominent nucleus and increased cytoplasmic granularity. Most notably, these cells become sensitive to a staining technique that visualizes senescence-associated β-galactosidase (SA-β-gal) activity (12). This technique, which stains perinuclear compartments blue, is a widely accepted and utilized marker of senescence (Figure 1).

Studies have established that senescence-associated mechanisms prevent cells from proliferating indefinitely *in vitro* and that immortalization circumvents this tumor suppressor mechanism (7). More recently, senescence tumor-suppression has been identified in non-malignant human growths *in vivo*. Benign melanocytic nevi (e.g. skin moles) result from the increased activity of mutant B-Raf (13). After increased proliferation and growth, melanocytes arrest growth, increase expression of $p16^{Ink4a}$ and stain positive for SA-β-gal. Increased expression of CDKI proteins and increased SA-β-gal staining is also observed in lung adenomas, but not in adenocarcinomas, suggesting that senescence suppresses malignant transformation (14). Senescence has also been also identified in more extensive cases of benign prostatic hyperplasia (BPH), and prostatic intraepithelial neoplasia (PIN), both non-malignant conditions (15, 16). These data demonstrate the role of senescence as an endogenous barrier to malignant transformation, and the identification of senescence in tumors may suggest a more benign or favorable outcome.

It is of clinical interest that transformed cancer cells can be induced to a similar senescent state both *in vitro* and *in vivo* with several conventional anti-cancer treatments. Senescence has been identified in tumors after radiation or genotoxic chemotherapy (10, 17, 18), although this appears to be inefficient with currently available therapies. This response is often induced by lower doses of cytotoxic agents *in vitro* (9). As a therapeutic goal, senescence may provide an effective means to induce persistent cytostasis in both early and late stage cancers while limiting toxicity. Moreover, some evidence suggests that senescence may

function as a “back-up” response to therapy in cancer cells in which apoptotic pathways are disabled (19).

In this review, we discuss current information on treatments that induce senescence in cancer cells and the molecular pathways that regulate these events. Experimental evidence indicates there are benefits to the induction of senescence in immunocompetant patients. Finally, we will examine markers and other characteristics by which cellular senescence may be identified in tissue samples, and their potential prognostic uses.

2. The genetics of senescence induction

The basic opposing relationship of senescence and oncogenic transformation is central to senescence tumor suppression induced by both endogenous processes and as part of a treatment strategy. In the 1980s, early genetic studies of senescence were performed by fusing mortal cells, which retain the capacity to become replicatively senescent, and immortalized transformed cells (20-23). This resulted in cells with limited replicative capacity demonstrating that immortalization occurs by “turning off” or bypassing senescence-inducing genes and pathways and that active processes are replaced or re-engaged to induce senescence in the hybrids. In a series of elegant experiments, Pereira-Smith and Smith found that fusion of specific cancer cell lines results in mortal hybrids and that these cells could be placed into one of four genetic complementation groups (22). Importantly, these experiments suggest that four genetic pathways regulate senescence and that only one deficient process needs replacement to reactivate senescence in cancer cells.

While the identity of these molecular pathways has yet to be completely defined, several chromosomes (e.g. 4 and 20) and distinct genes have been found to induce senescence when reintroduced in cancer cell lines (Table 1). A number of these genes are known to have growth inhibitory functions, and include p53 and Rb (4, 5, 24). Cells with functional Rb and p53 appear more sensitive to stress and oncogene activities that stimulate senescence (7, 25). However, it

is significant from a therapeutic standpoint that cancer cells containing mutant or inactivated Rb or p53 retain the capacity to be induced to senescence. In separate studies of Saos-2 colon and DU145 prostate cancer cells, both lacking Rb and p53, doxorubicin induced senescence *in vitro* (9, 11). This indicates the presence of p53- and Rb-independent senescence activating pathways may be induced in cancer (Figure 2). These pathways appear to involve stress- and damage-response signaling mechanisms that directly affect gene transcription without the direct involvement of these classic tumor suppressor genes.

A number of genes, not necessarily related to p53 and RB, are active during senescence and induce senescence when overexpressed in selected cancer lines. These include the CDKI proteins $p16^{Ink4a}$, $p21^{Waf1/Cip1}$, and $p27^{Kip1}$ (5, 8). We have demonstrated in prostate epithelial and urothelial cells that $p21^{Waf1/Cip1}$ appears more important in the cell cycle arrest associated with early senescence, while $p16^{Ink4a}$ is more central to maintaining this phenotype (26). The p53-related proteins p63 and p73 also regulate senescence induction by similar mechanisms to p53 (4, 27-29). Tumor cell senescence has also been induced by IGFBP-rP1, a member of the insulin-like growth factor protein family (30). Other related genes are overexpressed during senescence (e.g. IGFBP3, IBFBP5, IGFBP7) suggesting the IGF-pathway is intimately involved in regulating both proliferation and senescence (31-33). Suppressing apoptosis by expression of the pro-survival Bcl2 has also been found to induce senescence, dependent on $p27^{Kip1}$ expression (19, 34). These studies suggest an intricate balance exists between proliferation, apoptosis and senescence that might be further exploited therapeutically.

3. Mechanisms and timing of senescence induction in cancer cells

The known mechanisms by which drugs and other therapies induce senescence revolve primarily around genomic stress and typically take several days to develop the full phenotype. This is in contrast to apoptosis which is invoked rapidly and has evolved to a large extent in less than 24 hours. Rather than the rapid activation of apoptotic processes that commit the cell to

destruction, senescence requires active changes in gene expression that mediate the molecular and cellular changes that define the senescent phenotype. Senescence both *in vitro* and *in vivo* does not fully develop its characteristic expression of SA-β-gal and morphology for 3-7 days after exposure to an agent (7, 9, 11, 18). This is accompanied by patterns of increased and decreased gene transcription that ultimately leads to stable senescent cells (35).

The cellular decision between apoptosis and senescence is dependent, in part, upon the magnitude of stress to which cancer cells are exposed; lower levels of damage may trigger senescence-associated, anti-proliferative responses without activating the cascades of caspase activity that commit the cell to apoptosis(9). While similar stresses can induce both apoptosis and senescence, the regulation of these processes are distinct and these pathways diverge. This is supported by studies showing senescence results when apoptosis is blocked by the overexpression of Bcl2 or the inhibition of caspases (19, 34, 36). These data imply that the point at which these pathways diverge occurs upstream from caspase activation. Identification and characterization of this regulatory nodal point is an area of active research interest.

Most senescence-inducing drugs are thought to generate DNA damage through various mechanisms to produce single- and double-strand breaks. Other agents have been shown to induce senescence through mechanisms that alter normal DNA structure and function without directly causing breaks or damage to DNA. These include the DNA methyltransferase inhibitor 5-azacytidine, which inhibits DNA methylation, and Sirtinol, a histone DNA acetylase inhibitor that alters normal chromatin structure (11, 37). Oxidative stress is closely associated with senescence induction both in normal and immortal cells (38). In addition to the oxidative damage to DNA, oxidative stress may also oxidize lipids, proteins and affect mitochondrial function. The Zn^{2+} ionophore pyrithione is thought to generate oxidative stress, leading to growth arrest and senescence (39, 40). Interestingly, the diterpene esters TPA and natural products PEP005 and PEP008, which activate PKC and MAPK activities, have been shown to induce senescence (41, 42). This may occur either as a direct result of increased PKC

signaling activity or due to genomic stress that results from pro-mitotic signaling, but these issues have not been thoroughly investigated. Regardless, the activity of these drugs implicates PKC signaling in the regulation of senescence induction. In all, senescence inducing drugs may potentially share common mechanisms affecting either genomic integrity or signaling responses that result from genomic stress.

4. Agents capable of inducing senescence in cancer

Observations that some tumors cells can be forced into senescence by agents used in the management of human cancers are of clear clinical interest (Table 2). These findings indicate many cancer cells possess intact, but silenced, signaling pathways that can be induced to stimulate senescence. This ability of tumor cells to undergo senescence in response to stress and damage has been noted with both radiation and chemotherapeutic drugs (4, 10). When equitoxic levels of different agents were applied to HT1080 fibrosarcoma cells *in vitro*, the strongest induction of the senescent phenotype (based on SA β-gal and cell cycle analysis) was seen with the DNA-interactive agents doxorubicin and cisplatin (9). Lesser responses were observed with ionizing radiation, etoposide and the lowest with the microtubule-targeting drugs docetaxel and vincristine. Furthermore, drugs such as doxorubicin induced senescence in numerous cancer cell lines, including those lacking p53 and $p21^{Waf1/Cip1}$(9, 43). We have shown that prostate cancer cell lines express different CDKIs at senescence when compared to primary prostate epithelial cells suggesting that compensatory mechanisms are involved in cancer cell senescence (26). While senescence induction remained inefficient for many drugs, occurring in only a subset of treated cells, these results suggest that damage responses that signal senescence remain competent in cancers lacking major tumor suppressors.

A limiting factor in the identification of new compounds that induce senescence efficiently has been the lack of methods to rapidly screen compounds and small molecules. Until recently, the senescence-inducing activity of compounds was evaluated individually in a

time-intensive and focused manner. In response to this, our laboratory developed a method to screen small molecule and other compound libraries for senescence activity using a robotic fluid handler and plate reader (40). This whole cell assay is based on identifying several characteristics of senescent cells including the development of permanent growth arrest, characteristic senescent morphology and positive SA-β-gal staining. This method was used to screen a 4160 compound library of known bioactive compounds and natural products at a 10μM dose. Four lead compounds not previously associated with senescence were identified for further investigation. One agent, the quinone diazequone (AZQ), had been found to induce tumor stasis in experimental solid tumor models in the 1980's, but was not pursued further because of the lack of tumor regression (44, 45). Although further work needs to define the mechanisms by which these compounds induce senescence, some are known to induce DNA alkylation and oxidative stress at high doses, which are closely associated with senescence. The mechanisms induced by other identified compounds may involve novel targets including post-translational modifications involved in proteolytic processing and kinase signaling. Further utilization of this approach should permit the identification of additional senescence-inducing agents, as well as provide additional tools to understand the molecular basis for this response.

Significantly, senescence has been identified in patient tumors removed after genotoxic treatment. Areas of increased SA-β-gal staining were observed in 41% of breast tumors after treatment with a regimen of cyclophosphamide, doxorubicin, and 5-fluorouracil (18). This staining was confined to tumor cells with no detection in normal tissues. Senescence markers have also been observed in lung tumors after treatment with carboplatin and docetaxel (17). Despite the relative inefficiency of these regimens for inducing senescence, these studies suggest that senescence may be a more prevalent tumor response to current anti-cancer therapy than previously realized.

5. Cellular Senescence: Friend or Foe?

The therapeutic induction of senescence in tumor cells induces several features that may be beneficial to the treatment of cancer. Importantly, senescence stimulates a persistent terminal growth arrest. Cells remain viable but are typically arrested at G1 or G2/M phases of the cell cycle and fail to proceed even upon mitogen stimulation (7). This is in part due to the increased expression of one or more cyclin dependent kinase inhibitors including $p16^{Ink4a}$, $p21^{Waf1/Cip1}$, and $p27^{Kip1}$ (8). These cells may persist indefinitely in a stable state even *in vivo*. Senescent melanocytes have been identified in benign nevi that remain for years (13). We have recently demonstrated the persistence of senescent prostate cancer cells at least 6 weeks after the establishment of xenografts with doxorubicin-induced senescent prostate cancer cells (46). Alternately, senescent cells may survive over prolonged periods before becoming non-viable *in situ* and undergoing phagocytosis. The enrichment of lysosomal β-galactosidase activity with the development of the senescent phenotype suggests that some senescent cells may eventually undergo autophagy (47).

The presence of senescence in tumor cells can stimulate an immune response. In melanoma co-expressing N-Ras and B-Raf mutants, senescence increased these cells susceptibility to cell-mediated cytotoxicity *in vitro* by lymphokine-activated killer cells (48). This response was also observed in a mouse hepatocarcinoma model in which the conditional expression of functional p53 induced senescence lead to tumor regression mediated by an innate immune response (49). This may be instrumental in the prolonged survival found in a mouse lymphoma model in which senescence was induced by chemotherapy and Bcl2 expression (19). However, this benefit has yet to be specifically investigated in therapeutically-induced senescent tumor models. Such studies are limited by the requirement for immune-deficient mice when using human xenografts, and the lack of compounds that specifically induce senescence in endogenous tumors *in vivo.*

Another potential benefit of therapeutic senescence is the observation that lower concentrations of many drugs induce this response in contrast to higher doses which lead to

apoptosis or necrosis. We recently screened a series of concentrations of doxorubicin, 5-azacytidine and docetaxol for their ability to generate senescence (11). At lower doses the senescent phenotype was predominant, albeit inefficient in inducing senescence in most prostate cancer cell lines. Higher doses that lead to elevated DNA damage and stress are associated with a more pronounced apoptotic response. This suggests that induction of senescence in tumors may be achieved with lower drug doses, perhaps administered chronically, potentially limiting treatment-related toxic side-effects.

Other features associated with senescence have generated concern for oncologists. One is the idea that senescence may be a reversible process, at least in senescent fibroblasts, if proteins involved in its maintenance are lost. The overexpression of simian virus 40 large T antigen protein or the inactivation/downregulation of p53 and $p16^{Ink4a}$ result in proliferation in senescent fibroblasts (25, 50). However, these induced cells had a limited proliferative capacity undergoing only a few cell divisions before becoming apoptotic. Notably, whether drug-induced senescence is a reversible process has not been addressed experimentally. However, it appears unlikely that the expression of proteins that block proliferation, notably the CDKIs, and the extensive changes in nuclear structure could be reversed in senescent patient tumors.

Senescence in fibroblasts may result in the resistance of these cells to programmed cell death. Senescent fibroblasts resist the apoptotic effects of serum starvation (51) and hydrogen peroxide (52). However, senescent human umbilical vascular endothelial cells are more prone to apoptosis than fibroblasts, suggesting this phenomenon is cell-type specific (53). DeJesus *et al* found that pro-apoptotic signaling via ceramide and TNF-α is interrupted in senescent fibroblasts and may be a mechanism by which apoptosis is avoided in these cells (54). To date, the resistance of cancer cells to apoptosis after senescence induction has not been clearly addressed.

Studies in the aging field have suggested an association between age-related senescence and the promotion of carcinogenesis in surrounding tissues (55). Senescent

fibroblasts secrete characteristic pro-inflammatory immune cytokines, including IL6 and IL8, that have the potential to promote bystander cell proliferation and may account for the development of some age-related cancers (56). While this may mediate senescence-related effects of aging, the relevance of this phenomenon to treatment-induced senescence in cancers is unclear. We and other researchers have demonstrated that senescent gene expression patterns vary markedly between fibroblasts, epithelial cells and cancer cells (31, 46, 56, 57). This would imply that each cell group would have different effects on bystander cells

Recently, we examined the effect of doxorubicin-induced senescent prostate cancer cells on the growth of surrounding proliferating cancer cells (46). In a series of experiments we demonstrated that over time tumor growth was not affected by increasing numbers of senescent cells and that the proliferation rate of these bystander cells was not increased. A subsequent publication found an antiproliferative effect *in vitro* using MCF-7 breast cancer cells induced to senescence by adriamycin/doxorubicin (58). Finally, one of the most notable findings suggesting senescent cells have no impact on proliferation is the observation that benign nevi containing senescent cells persist chronically for years, yet remain uniformly benign and stable (13). This issue remains an area of debate. The shorter timescale of exposure to senescent cancer cells in patients makes it less likely that a major bystander effect of senescence induction is proliferation. Ultimately, the effects and consequences of senescence induction as a therapeutic strategy may vary with the cancer type as well as the drug utilized to induce senescence.

6. The identification of senescence *in vitro* and *in vivo*

The ability to identify markers associated with senescence is an important aspect for the utilization of this phenotype in clinical practice. Senescence has been routinely identified by staining techniques visualizing SA-β-gal activity (12). This has been used as a marker in aging tissues (12) and in tumor tissues after chemotherapy (17, 18). SA-β-gal staining is dependent

on increased lysozomal activity and requires fresh or frozen tissue for staining. Thus, this technique is incompatible with many immunohistologic techniques routinely used in clinical pathology laboratories. The gene associated with SA-β-gal activity, the lysosomal β-galactosidase *Glb1,* is not required for senescence growth arrest and may be uncoupled from senescence in some cancer cell lines (59). The development of immunohistologic methods to detect Glb1 protein expression and localization in paraffin-embedded tissue, while not improving the reliability of this marker, would nonetheless facilitate its use in archival samples.

To augment the identification of senescent cells *in vitro*, specific phenotypic characteristics can be useful (Table 3). When cells enter senescence they develop a distinctive morphology, becoming enlarged, flattened, and multinucleated (Figure 1). This morphology, however, is most easily identified *in vitro* and may not be apparent in tissue. Many senescent cells also develop extensive vacuoles in the cytoplasm, which may be associated with an increase in cellular complexity measured by flow cytometry as increased side scatter (SSC) (4). However, the most important characteristic of senescence is the irreversible loss of cell proliferative capacity. Cells accumulate in G1 or G2/M and S phase typically decreases in flow cytometric cell cycle profiling. In addition, cells become multinucleated, identified by the occurrence of additional 2N and 4N peaks. These analyses may provide objective data used to identify the senescent phenotype in patient samples.

Other markers focus on the localization of proteins involved in senescence signaling and unique morphologic changes that occur in senescence. Senescence-associated heterochromatic foci (SAHF) are condensed regions of heterochromatin that accumulate during senescence (60, 61). These foci are composed of methylated and deacetylated histones and other associated proteins. SAHF have been used to identify senescence *in vitro* in fibroblasts and other non-immortalized cells. More widely tested and specific markers in this category include H3K9 and H3K27 which co-localize in SAHF. In cancer, SAHF has been used to identify senescence in MCF7 cells (62). However, in cancer cells where chromatin patterns are

dysregulated, the occurrence and composition of these foci may vary. The utility of these markers to identify senescence in patient tissues is as yet unexplored.

Additional markers include CDKIs whose increased expression mediates cell cycle arrest. Amplified expression of the CDKIs $p16^{Ink4a}$ and related Ink4 proteins, $p57^{Kip2}$, $p21^{Waf1/Cip1}$, and notably $p27^{Kip1}$ have been seen in senescent cells and tissues (7, 8). However, some of these CDKIs can be inactivated in cancer cells making them less reliable markers. In cancer cells the downregulation of $p27^{Kip1}$ and expression of its regulator ubiquitin ligase Skp2 have been identified in prostate and other cancers (63, 64) as well as precancerous lesions (16). The $p27^{Kip1}$ gene is infrequently mutated or deleted in cancers, suggesting its induction may represent a more promising marker of senescence.

Senescent cells in tumors may also be identified by the increased expression of secreted proteins. Studies have shown that senescent cells increase the expression of a variety of secreted protein factors. These include proteins involved in IGF signaling (including IGF2, IGFBPs 3, 5, 6 and 7)(31-33, 43), immuno-inflammatory cytokines (e.g. IL6, IL8 and related proteins)(56, 65, 66) and CXCL14/BRAK whose function remains largely undefined (11). The induction of these secreted factors in senescence may potentially serve as serum-based markers for the identification of patients undergoing senescence responses.

Our lab has screened a series of genes upregulated during epithelial senescence for their role as markers of senescence in cancer (11). In a series of cancer lines using a number of senescence-inducing drugs, transcripts of *Cspg2* (versican), *FILIP1L* (previously *downregulated in ovarian cancer -1*), and *P311* genes were found to represent specific markers of senescence that are not induced during apoptosis. Changes in mitochondrial architecture may also be used as a marker of senescence. Mitochondria in proliferating fibroblasts are distinct and small, whereas in senescent cells mitochondria fuse into elongated and integrated networks (67). The expression and localization of the integral mitochondrial proteins hFis1 and OPA1 regulate these changes and the development of senescence (67). Finally, the genes

Dec1 (BHLHB2) and DcR1 (TNFRSF10D) have been associated with senescence in non-cancer tissues (14). While these proteins may be detected by immunohistochemistry, the utility of these potential markers to identify senescence in fixed patient tumors has yet to be investigated.

In summary, the most widely utilized marker of senescence is SA-β-gal, which when used *in vitro* with increased SSC, accumulation in G0/G1 and G2/M, and morphology provides strong evidence to identify senescence. Other useful markers include the CDKIs $p16^{Ink4a}$ and $p27^{Kip1}$, Cspg2 and FILIP1L, and the increased expression of secreted cytokines. *In vivo*, SA-β-gal staining in conjunction with CDKI protein induction and other markers of decreased proliferation provide powerful evidence for the presence of senescence. We emphasize that the identification of multiple markers in tissues provides more reliable evidence for senescence than that provided by a single marker.

7. Clinical Potential for Senescence-Based Tumor Suppression

Therapeutic senescence is a potential mechanism to induce cytostasis in cancer. The goal of this strategy is to inhibit tumor growth rather than to cause regression or ablation. Based on *in vitro* and xenograft studies this can be achieved by the chronic administration of low doses of senescence-inducing drugs (unpublished data). Effective dosing to achieve senescence will vary with the drug, but would likely involve lower doses than those that generate apoptosis (40). Notably, tumor models with inactivated apoptotic signaling pathways respond robustly to senescence-inducing drugs, leading to improved survival after chemotherapy (19). As with many anti-cancer compounds, tumors might produce a heterogenous response to senescence-inducing therapy. However, recent data suggests the expression of cytokines and secreted factors may have an inhibitory effect on the growth of surrounding cells (33, 58, 65, 66), resulting in inhibitory or growth-neutral effects on bystander cancer cells (46, 49, 58). With the further identification of precise pathways that trigger senescence, and additional specific

senescence-inducing agents, we foresee a wider exploitation of this approach in cancer treatment.

Several scenarios can be anticipated for the utilization of senescence in clinical cancer therapy. One is its use in clinically advanced tumors. These cancers frequently contain cells that have bypassed senescence-associated barriers associated with oncogenic progression (5). However, data from our laboratory and others indicate that even in these advanced cancers senescence can be induced utilizing specific drugs (9, 11). Tumor tissues may be monitored for changes in size, senescence activity (SA β-gal, $p21^{Waf1/Cip1}$), and other markers of proliferation such as KI67. Given the robust secretory activity of senescent cells, serum markers of senescence (e.g. BRAK(CXCL14), IL8, CSF, IGFBPs) might be utilized to measure response. An alternate use for senescence-inducing compounds would be the treatment of pre-malignant or early cancer. Research has suggested that cells in the premalignant prostatic lesion PIN are frequently senescent (15, 16), resulting from extended DNA replication, cellular damage or endogenous stresses. This may reflect a predisposition and sensitivity of prostatic epithelium to senescence-induction and drug-induced senescence. Retinoids have demonstrated senescence-inducing activity in a number of cancers and have demonstrated chemopreventative effects for a number of cancers with few side-effects [roninson]. This observation of senescence in endogenous premalignant and malignant cells provides an opportunity to selectively treat cancer cells while minimizing toxicity in normal, nontumor tissues.

An alternative use of senescence could be as a marker for tumor prognosis. Given senescent cells display persistent growth-arrest, the presence of senescence in a cancer may indicate slower overall tumor growth or decreased metastatic potential. This relationship is illustrated by the identification of senescence markers in nevi, lung adenomas and other non-malignant growths including BPH and PIN (13-16). Lung adenomas express senescence markers such as SA-β-gal that are not found in adjacent cancers. Other markers associated with senescence, including $p27^{Kip1}$ and $p16^{Ink4a}$, are selectively expressed in cancers and their

presence is associated with improved prognosis and lower probability of relapse after treatment (68). A similar finding has been demonstrated for IGFBP3 and maspin in prostate and other cancers (11, 43, 69). Our lab and others have noted that a subpopulation within proliferating tumor cell lines (LNCaP, DU145, PC3, MCF7, HCT116 and others) appear to senesce spontaneously (9, 11, 17). This also occurs in patient tumors *in vivo,* with sporadic SA-β-gal staining observed in 1 of 3 lung tumors and 2 of 20 breast tumors in untreated control patients, while no staining was observed in normal tissue of any patient (17, 18). This intriguing observation suggests that pre-malignant and some tumor cells may spontaneously become senescent due to their inherent genomic instability or other factors. The detection of senescence in tumors may be indicative of increased genomic instability within the cancer suggesting greater vulnerability to genotoxic drugs. This finding might be exploited therapeutically to augment senescence induction in cancer cells.

8. Conclusions

In the war on cancer, the focus has been on achieving complete cure through eradication of the tumor. However, tumor cells are typically heterogeneous and adapt rapidly to toxic chemicals and varying environments. Increasing data supports an approach that incorporates the induction of senescence in cancer therapy. An understudied area of tumor biology suggests that the presence of cancer cells sensitive to therapy may suppress the growth of resistant clones (70). Furthermore, the obliteration of these sensitive cells using cytotoxic chemotherapy may result in the rapid, unchecked proliferation of resistant clones. Approaches designed to maintain a stable tumor volume may actually lead to improved survival (70). Given the toxicity associated with increased chemotherapy dosing and the development of these resistant clones, a strong rationale can be made for the further examination of senescence induction as cancer therapy.

The therapeutic induction of senescence is a potential means to treat cancer through induction of a persistent cytostatic state in tumors. In epithelial cells, senescence is an endogenous mechanism to limit the growth of non-malignant neoplasias. Accumulating data indicates cancer cells that have bypassed many major tumor suppressor blocks remain sensitive to induced-senescence, suggesting widespread utility in therapy. Other advantages of senescence include cytostasis, lower toxicity-related side effects, and immune stimulation. The finding that endogenous senescence and its markers indicate a favorable prognosis is an observation that must be investigated fully. This suggests an increased vulnerability of cancers to senescence-inducing agents. Cancer therapy to date has focused on complete eradication with the expense of treatment related complications. The therapeutic induction of senescence results in chronic tumors that may allow more patients to maintain a fine line between quality and quantity of life. Given the expected increase in cancer with the aging population, we need to strongly consider senescence in our armamentarium to treat cancer patients as effectively as possible while maintaining the quality of their lives.

Figure and Table Legends

Figure 1. Proliferating and Drug-Induced Senescent PC3 Prostate Cancer Cells. Senescent cancer cells exhibit the characteristic morphology and increased SA-β-gal activity of senescent cells. PC3 cells were cultured in drug-free medium or in medium containing 250nM AZQ for 3 days followed by 2 days in drug free medium (40), fixing and staining (12). Cells were visualized under 200X magnification using phase contrast microscopy. Scale bar = 10μm.

Figure 2. Drug-Induced Senescence in Cancer Cells with Intact or Mutated Tumor Suppressors. A. Exposure of wild-type proliferating cells to damage and stress-inducing drugs stimulate the activity of major tumor suppressor pathways, including p53 and Rb, to induce senescence. B. In cancer cells lacking major tumor suppressor pathways nevertheless remain vulnerable to some senescence-inducing drugs, presumably due to the activity of alternative pathways.

Table 1. Genes That Induce Senescence When Expressed In Cancer Cells.

Table 2. Drugs That Induce Senescence In Cancer Cell Lines and Tumors.

Table 3. Cellular Characteristics and Molecular Markers of Senescence in Wild-Type and Cancer Cells.

Mechanism Gene		Function	References
Mitotic and Stress Signaling			
	Raf-1	Mitogenic/Stress Signaling	(71)
	MKK6/p38	Mitogenic/Stress Signaling	(72)
Major Tumor Supressors			
	p53	Transcription Factor	(73)
	p63	Transcription Factor	(74)
	p73	Transcription Factor	(74)
	Rb	Transcription Regulator	(75)
CDKIs			
	p21Waf1/Cip1	Kinase Inhibitor	(76)
	p16Ink4a	Kinase Inhibitor	(77)
	p57Kip2	Kinase Inhibitor	(78)
	p15Ink4b	Kinase Inhibitor	(79)
Mitochondrial Integrity and Function			
	OPA1	Mitochondrial Membrane Structure	(67)
Pro-Inflammatory Signaling			
	IL-6/CXCR2	Cytokine/Receptor	(66)
	IGFBP-rP1	Cytokine/IGF Signaling Modulator	(30)
	IGFBP7	Cytokine/IGF Signaling Modulator	(33)

Agent	
Aphidocolin	(9)
Bleomycin	(80)
Camptothecin	(81)
Carboplatin + Docetaxel	(17)
Cisplatin	(9)
Cyclophosphamide + Doxorubicin + 5-Fluorouracil	(18)
Diaziquone/AZQ	(40)
Doxorubicin	(9)
Epigallocatechin Gallate (EGCG)	(82)
Etoposide	(9)
Gamma Irradiation	(10)
Hydroxyurea	(83)
K858	(84)
Mitoxantrone	(56)
Pyrithione	(40)
Resveratrol	(85)
Retinols	(9)
TPA, PEP005, PEP008	(41, 42)
Lovastatin	**(86)**

Marker	Reference
Morphology	(4, 6)
SA-β-Gal Activity	(12)
Glb1	(59)
SSC	(4)
BrdU Incorporation	
DAPI/Hoechst 33342	(40)
Decreased KI-67	(14, 49)
Propidium Iodide Exclusion/Annexin V staining	(40) ?
Lack of Cleaved PARP	(40, 49)
Lack of Cleaved Caspase 2/3/9	
Lack of TUNEL Staining	(49)
$p16^{Ink4a}$	(8)
$p21^{Waf1/Cip1}$	(8)
$p27^{Kip1}$	(8)
DAPI/Hoechst 33342	(60)
HIRA	(60)
H3K9-$methyl^{3}$	(60)
HP1γ	(60)
IGF2	(31, 32)
IGFBP3, IGFBP5; IGFBP7	(31, 32, 43, 66)
IL-6, IL-8, CXCR2 and others	(56, 65, 66)
Versican/CSPG2	(11)
CXCL14/BRAK	(11)
Mitochondrial Fusion/hFis1/OPA1	(67)
Dec1	(14)
DcR2	(14)

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